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LOCAL SPECIFIC THERAPY OF EXPERIMENTAL PNEUMOCOCCAL MENINGITIS.

III. Incidental Myelitis, Abscess, and Organization of Exudates.

By FRED W. STEWART, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 1 to 4.

(Received for publication, October 26, 1927.)

During the past year our attention has been directed toward the study of Type I pneumococcal meningitis in the effort to devise some method of therapy which might be hoped to reduce the mortality in this disease. The results of these experiments are described in the first two papers of this series (1, 2). The purpose of the present article is to place on record, mainly photomicrographically, certain features in the pathology of experimental Type I meningitis, occurring late in the course of the disease in dogs, emphasizing thereby some aspects of the experimental infection, which are either rare or else for the most part unnoted in its human prototype.

It early became apparent that a considerable number of the experimental dogs developed central cord lesions. These lesions ranged anywhere from occasional leucocytes in the central canal of the cord, with slight leucocytic invasion of the ependyma and slight perivascular leucocytic infiltration, up through purulent distension of the central canal and adjacent perivascular channels, to, in some instances, widespread destructive myelitis. Similar conditions in human pneumococcal meningitis must be very rare, or else unnoted,—rare probably because the cases are too rapidly fatal to permit their development, or unnoted because cord examinations are infrequent at necropsy unless the clinical data question the possibility of special cord pathology.

Nevertheless, Florand and Nicaud (3) report a terminal pneumococcal myelitis, accompanied by a localized meningitis in a patient with pneumonia and empyema.

It is a noteworthy fact that crisis had occurred with the probable establishment of partial immunity 6 days before the nervous manifestations began, this partial immunity doubtless accounting for the localized character of the meningitis and its duration (25 days). The authors believe the myelitis to have been of vascular origin. Marinesco (4) was able to recover the pneumococcus from a case of myelitis, but we are left in doubt as to whether or not there was a preceding meningitis. Chiari (5) reports a "myelitis suppurativa" complicating bronchiectasis, but the protocol is more suggestive of multiple cord abscesses, possibly embolic in origin; pneumococci were recovered from the lesions.

Despite the apparent rarity of cord involvement, other than meningeal, in human pneumococcal meningitis, it is quite common in dogs. This statement is based upon the gross and microscopic examinations of spinal cords from twenty-six dogs; these animals in some instances were controls, where no treatment was instituted after infection; others died either from accident during treatment, from intercurrent distemper, or of the disease, in spite of treatment. Two were reinfected animals that died after prolonged disease induced by reinfecting the meninges some weeks after treatment had resulted in a cure of the primary infection.

The incidence and types of cord involvement in the series of animals examined are shown in Table I. Thus it is seen that seventeen out of twenty-six dogs had purulent material within the central canal of the cord; this consisted sometimes of a few polymorphonuclear and endothelial leucocytes in a mass of precipitated albumin, with occasional strands of fibrin; in other instances the central canal was markedly distended with pus (Fig. 1), so much so that in the fresh gross specimen this pus could be easily expressed by gentle stroking. Paralleling almost, but not quite, the occurrence of pus in the central canal, was a varying degree of leucocytic invasion of the ependyma and of the gray matter immediately surrounding the canal and less often a perivascular infiltration of adjacent vessels; with purulent distension of the canal the perivascular involvement was very marked (Figs. 1 and 3). These cord lesions were found only once in animals dying previous to 42 hours after infection. Severe purulent myelitis with extensive destruction of the spinal cord occurred in but four animals; one of these died at 44 hours; the others succumbed late in the disease. Curiously enough, the severe myelitis in all instances spread into the dorsal half

of the cord, destroying especially the dorsal cornua and only slightly involving the ventral gray matter. Only once did this lesion occur in a treated animal; hence the possibility of its arising from cord trauma is very remote, since no animals were infected subthecally in the cord

TABLE I.

Dog No.	Hrs. after injection	Pus in central canal of cord	Infiltration of region of commissures	Perivascular infiltration	Severe myelitis
1	144	+	+	+	0
2	120	0	+	+	0
3	192	+	+	+	+
4*	114	+	+	+	+
5	90	+	+	0	0
6	90	+	+	0	0
7*	216	+	+	+	+
8	42	+	+	+	0
9	216	+	0	+	0
10	90	+	+	0	0
11	23	0	0	0	0
12	44	Severe myelitis; no trace of central canal			
13	42	+	+	+	0
14	24	0	0	0	0
15	43	Traumatic abscess of cord			
16	114	+	+	+	0
17	90	0	+	0	0
18	16 days	+	0	0	0
19	114	0	+	+	0
20	162	+	+	+	0
21†	288	0	0	0	0
22	42	+	+	+	0
23	114	+	0	0	0
24	42	0	0	0	0
25	66	+	+	+	0
26	18	+	+	+	0

*Reinfected animal.

†Meninges sterile.

region. Conditions are best described by reference to the photomicrographs.

Figs. 1 and 3 are from animals untreated after cisternal infection. The first was a reinfected dog, whose second infection followed 11 weeks after treatment had resulted in the cure of the primary infection.

This reinfected animal survived 8 days, eventually becoming moribund, at which time it was chloroformed. The second dog was given a weak primary infection, remained untreated, and died of the disease on the 8th day. Both cords microscopically show considerable dilatation and are filled with an exudate of polymorphonuclear leucocytes; in one the ependyma is invaded by leucocytes, but is otherwise intact; in the other the ependymal cells are flattened and partially absent and leucocytes are invading the cord parenchyma adjacent to the ependyma. In both cords the blood vessels near the central canal show well marked perivascular accumulations of leucocytes, mainly polymorphonuclears. As invasion of the parenchyma continues, the nerve cells undergo extensive necrosis and are invaded by polymorphonuclear and endothelial leucocytes, the latter becoming large and vacuolated, probably from the ingestion of fatty materials (Fig. 6); the central canal may be completely obliterated by the process in some regions.

Figs. 2, 4, and 5 are from the same cord as Fig. 1; here, however, the process has resulted in what is essentially an abscess burrowing extensively throughout the dorsal gray matter, but only slightly involving the fiber tracts. In certain cords pneumococci have been demonstrated in the sections; indeed, in one instance they were numerous in the gray matter surrounding the central canal and in the canal itself before any significant degree of cellular reaction had manifested itself. Briefly summarized, it may be stated that nearly all dogs suffering from experimental Type I pneumococcal meningitis and surviving beyond the 2nd day of the disease develop some degree of central cord involvement. Such central lesions may progress toward frank suppurative myelitis, and thereby provide foci of disease in no wise amenable to treatment.

Abscesses appear to be uncommon in human pneumococcal meningitis, although cognizance is, of course, taken of the fact that abscesses, for example, of otitic origin, may initiate the process; when we speak of abscesses, we refer to those *following* a primary meningitis. That such may occur, however, is apparent from the reports of Kolmer (6) (cerebellar abscess 4 weeks after meningitis was thought to have been cured) and Wieder (7) (frontal lobe abscess). In the dog abscesses are rare. Fig. 7 shows an abscess of recent origin in the floor of the IVth ventricle; the animal was a reinfected dog and was untreated. Small

cortical abscesses are occasionally seen; they arise either as direct extensions from the meninges *via* the perivascular sheaths, or in infected purpuric hemorrhages; this cortical purpura is a common finding and probably, as a rule, has little significance in abscess formation; for the most part it merely results in collections of endothelial leucocytes filled with blood pigment, the usual reaction to hemorrhage.

It is of interest to inquire into the ultimate fate of the meningeal exudate in animals dying late in the course of the disease, to determine whether or not there is any tendency toward resolution. In all dogs examined, with two exceptions, the process must still be regarded as acute; the two exceptions are animals with fatal reinfections. In both of these animals there is distinct evidence of organization of exudate; this organization is coincident with a change in character of the cellular reaction and the appearance of many lymphocytes, plasma cells, and phagocytic endothelial leucocytes. The fibrin network thickens and shrinks and is invaded by endothelium and fibroblasts (Fig. 9); this organization by endothelium and fibroblasts parallels in intensity the distribution of fibrin. The appearance of many chronic inflammatory cells in the exudate produces a very peculiar alteration in sections, particularly in the regions of the choroid plexuses, superficial penetrating vessels, and occasionally in the central cord lesions; there is little or no increase in the actual number of vessels, but a tremendous perivascular thickening well shown in Fig. 8, where every vessel in the choroid plexus is surrounded by a darkly staining zone of chronic inflammatory cells, outside of which the acute character of the exudate is still apparent.

In these chronic, perivascular, inflammatory zones may be observed a decided increase in what Kubie (8) describes as the clasmatocytes of the adventitial sheath, which he has found to multiply under conditions of stimulation, such as, for example, herpetic encephalitis. This clasmatocytic stimulation produces marked perivascular thickening; in certain vessels the clasmatocyte increase appears to be independent of the vascular endothelium, the latter being traceable as a thin, endothelial lining beneath thickened layers of sheath clasmatocytes; in other instances the endothelium seems to take part in the process and pictures such as that shown in Fig. 10 result, where no distinction appears between the endothelium and perivascular cells, suggesting,

therefore, that the swollen endothelial cells may be contributing to those of the adventitial sheath. Such supposition gains confirmation from numerous examples of processes, such as those apparent in Figs. 13 and 14, the former showing a diaster in the thickened endothelial cell, with surrounding perivascular clasmatocytic increase, the latter displaying two mitoses, one outside of the endothelium, the other apparently splitting off toward the vascular channel. Figs. 11 and 12 both demonstrate mitoses in perivascular spindle-shaped cells, closely applied to the endothelium, but nevertheless distinctly separate. Kubie was unable to detect the participation of endothelium in the increase of adventitial clasmatocytes, but the stimulation was doubtless less severe and the process less active in tissues studied by him.

SUMMARY AND CONCLUSIONS.

1. In experimental Type I pneumococcal meningitis of dogs central cord involvement is common after the 2nd day of the disease.

2. These central cord lesions may progress toward extensive suppurative myelitis.

3. Abscesses are infrequent; they arise either as direct perivascular extensions of the meningeal disease or at the site of infected purpuric lesions.

4. In prolonged untreated meningitis of reinfected animals, nothing resembling the disappearance of exudate following crisis in pneumonia occurs. The tendency is toward organization.

5. Late lesions are characterized by the marked development of a perivascular clasmatocytic apparatus, similar to, but more extensive than that hitherto reported in experimental herpetic encephalitis.

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4. Marinesco, G., *Nouv. iconog. Salpêtrière*, 1900, xiii, 561.
5. Chiari, H., *Z. Heilk.*, 1900, xxi, 351.
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EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Spinal cord. Distended central canal, destruction of ependyma, leucocytic invasion of gray matter in region of commissures. × 110.

FIG. 2. Acute suppurative myelitis. × 18.

FIG. 3. Spinal cord. Pus in central canal, invasion of ependyma, marked perivascular infiltration. × 130.

PLATE 2.

FIG. 4. Acute suppurative myelitis. × 18.

FIG. 5. A higher power view of the same process. × 160.

FIG. 6. Necrotic anterior horn cells, inflammatory cells, many fatty endothelial leucocytes. × 320.

PLATE 3.

FIG. 7. Abscess of medullary floor. × 18.

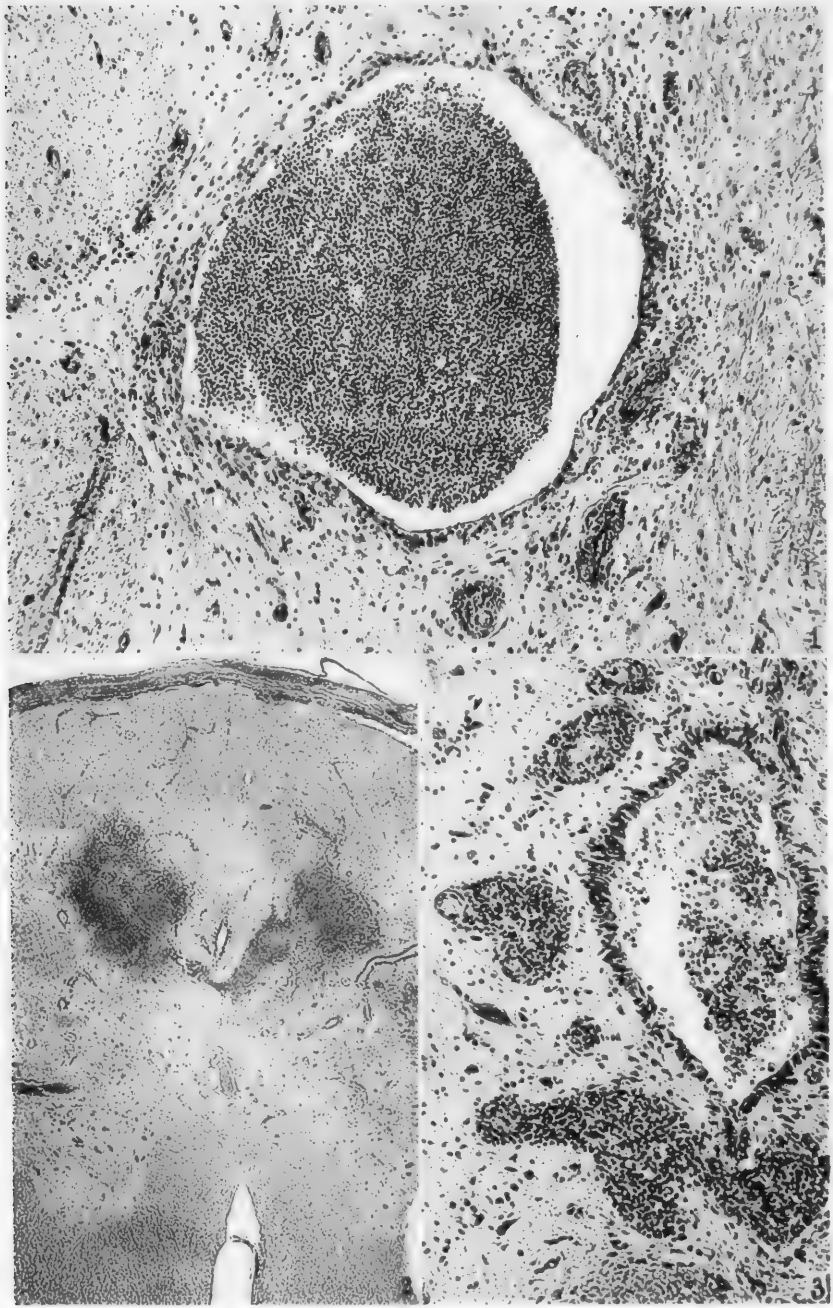
FIG. 8. Choroid plexus, showing marked perivascular thickening. × 18.

FIG. 9. Organization of fibrin in choroid plexus late in the course of a reinfection meningitis. × 570.

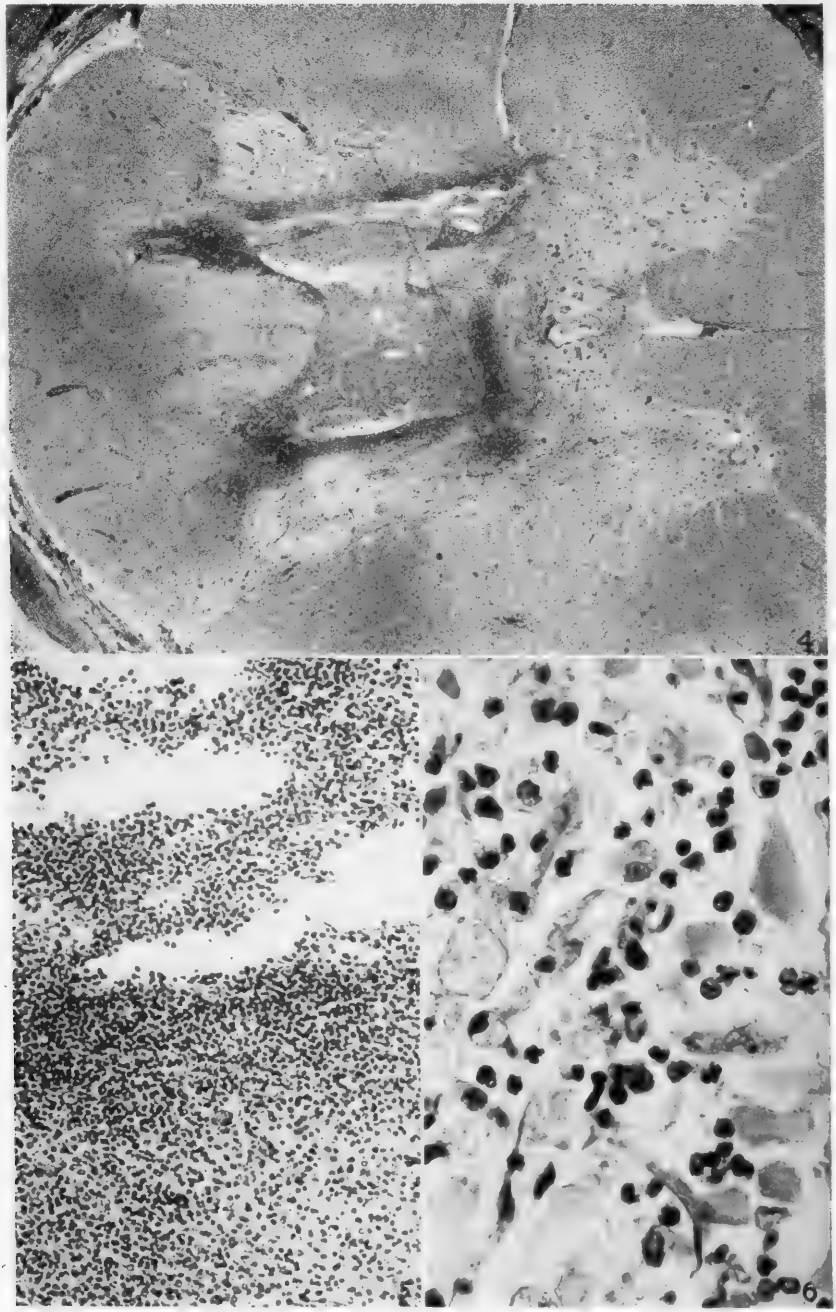
FIG. 10. Increase of adventitial clasmatocytes, suggesting a reduplication of the endothelium. × 1000.

PLATE 4.

FIGS. 11 to 14. Endothelial and perivascular clasmatocyte proliferation, giving rise to the thickened perivascular phagocytic apparatus. × 1000. (*a* = mitotic figures.)



(Stewart: Pneumococcal meningitis. III.)



(Stewart: Pneumococcal meningitis. III.)

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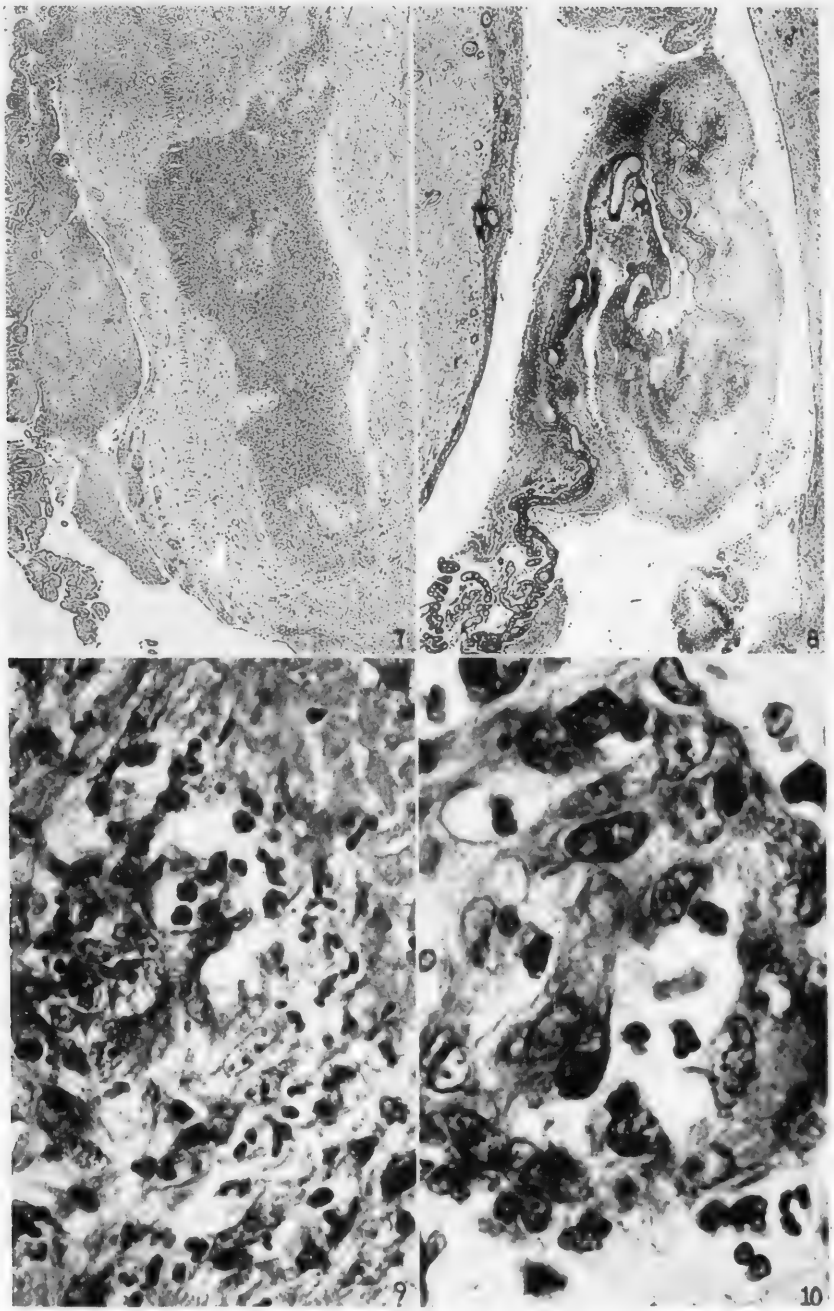

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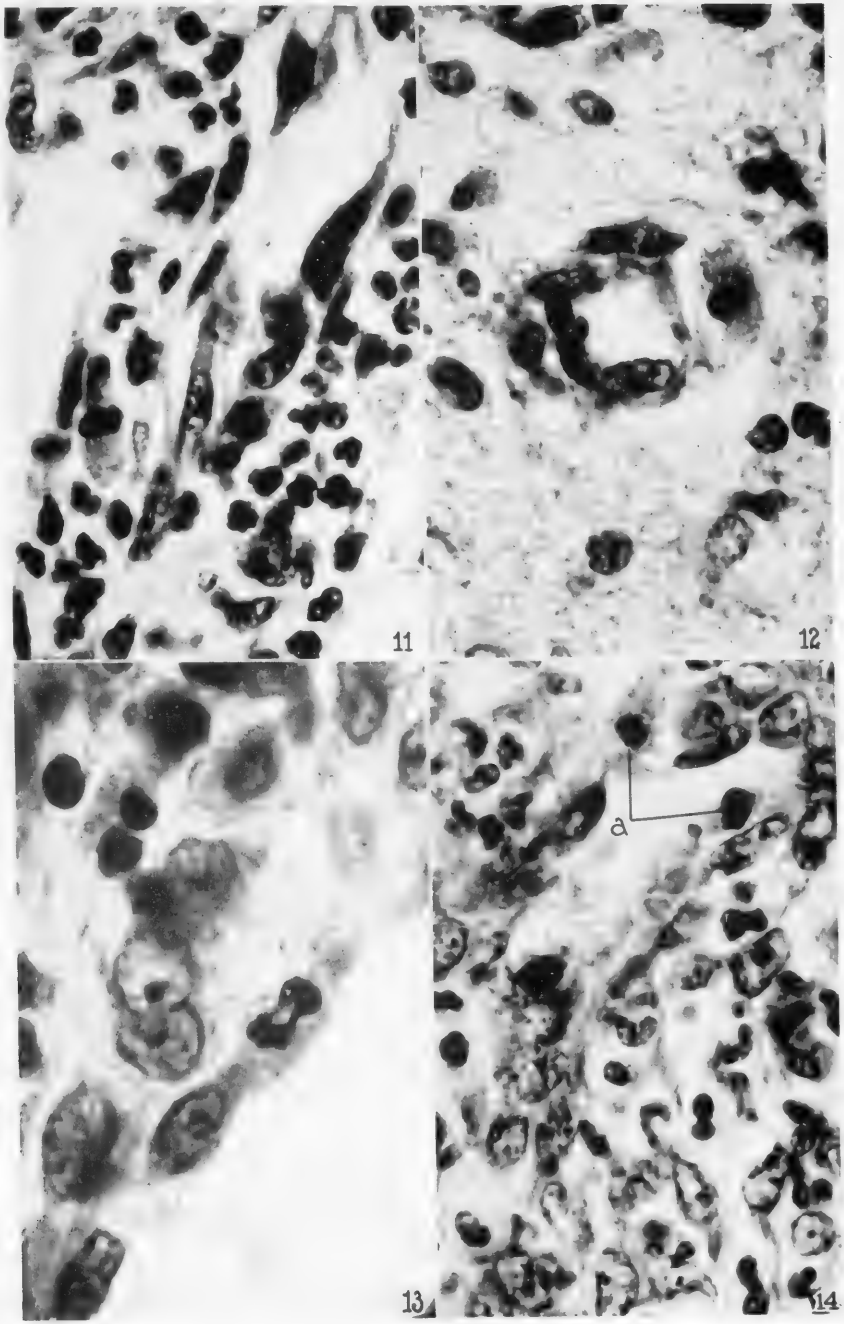

(Stewart: Pneumococcal meningitis. III.)

CONTRIBUTIONS TO THE PATHOLOGY OF EXPERIMENTAL VIRUS ENCEPHALITIS.

IV. Recurring Strains of Herpes Virus.

By SIMON FLEXNER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 28, 1927.)

Anyone wishing to inform himself of the present state of our knowledge of the subject of virus encephalitis[1] would do well to read the two comprehensive reviews of Doerr.[2] While these reviews deal chiefly with herpes virus, they touch on the related subject of so called encephalitis virus as well. In this way, the etiology of epidemic encephalitis is also brought under consideration. While Doerr inclines to the view that epidemic encephalitis in man is a form of herpes virus infection, yet he presents an impartial because quite complete discussion of the many sides of this important topic of controversy.

Our studies, to which those to be recorded in this paper relate, have dealt with the encephalogenic and other properties of the herpes virus, because Flexner and Amoss[3] were led to an investigation of this virus through experiments regarding the etiology of epidemic encephalitis. The conclusions reached were in conflict with those previously announced by Doerr[2] and by Levaditi.[4] The studies on the etiology of encephalitis have been continued and the present papers are intended to record the results of the more recent experiments. These later studies support the view previously put forward, namely that herpes virus encephalitis and epidemic encephalitis are definite pathological affections, etiologically distinct.

[1] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 231.

[2] Doerr, R., *Zentr. Haut- u. Geschlechtskrankh.*, 1924, xiii, 417; 1924–25, xv, 1, 289; 1925, xvi, 481; *Centr. Bakt., 1. Abt., Orig.*, 1925–26, xcvii, suppl., 76.

[3] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215, 233, 357. Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785.

[4] Levaditi, C., L'herpès et le zona, Paris, 1926.

I.

Persistent Strains of Herpes Virus.

The tendency for simple or febrile herpes to recur in certain individuals has often been noted. In these susceptible subjects, relatively trifling causes bring on a herpes of the lips or other parts. This circumstance has led to discussions and experiments concerning herpes virus carriage in normal persons, regarding which beliefs are still divided.[5] What does not seem to have been attempted is the determination of the quality of the herpes virus present at widely separated intervals in the herpes-susceptible individuals. In other words, no one has undertaken to answer the question as to whether one or more strains of herpes virus occur in these persons.

We have studied one habitual herpetic subject from this point of view. The subject is a victim of frequent common colds, with attendant labial herpes. The first examination, carried out 4 years before the one now to be reported, has already been recorded in previous papers.[3] The first H. F. strain of herpes virus was isolated by rabbit inoculation in March, 1922. The vesicular fluid, sterile for ordinary bacteria, was injected subdurally into etherized rabbits and gave rise to violent symptoms of virus encephalitis on the 6th day of incubation, followed by death. The brain of this rabbit provided the material for the series of experiments already described.[3] This strain of H.F. virus was highly potent; of fifteen rabbits receiving eye instillations, either with or without corneal scarification, all developed severe keratoconjunctivitis accompanied by symptoms of virus encephalitis, terminating in death. In four of the fifteen instances, glycerolated virus was inoculated.[6]

The second examination of the subject was made in March, 1926, or after an interval of 4 years. The virus isolated came in this instance also from a labial vesicle, but it was secured through corneal and not from a subdural inoculation. Two rabbits received a salt solution dilution of the clear vesicular fluid, free from ordinary bacteria, in the cocainized, scarified right eye. Inflammation quickly ensued; the

[5] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 361. Doerr, R., *Centr. Bakt., 1. Abt., Orig.*, 1925–26, xcvii, suppl., 80.

[6] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 239.

eyes were closed and vesicles appeared along the incisions within 24 hours. By the 4th or 5th day, the temperature reached 41.7°C., and was accompanied by circling movements to the right. As the local symptoms subsided, these movements ceased. Recovery of the animals followed and was complete. The virus thus obtained was used in the subsequent experiments and designated H.F. II.

Corneal Passages.

Cornea to cornea inoculation was carried out in a series of eight rabbits. The inoculum was taken from the inflamed eyes on the 4th or 5th day of the keratoconjunctivitis. The course of the infection and inflammation was severe and uniform in the first four rabbits of the series, and moderate in the second four of the series. Circling and high temperatures occurred only, or markedly only, in the first four passages. All the inoculated animals recovered completely and, as will appear, were tested later for immunity. In one instance only did the symptoms point to more profound involvement of the brain. The protocol of this experiment follows.

Rabbit.—Apr. 9, 1926. Right eye cocainized and cornea scarified with cataract knife dipped in salt solution containing scrapings from cornea of rabbit of 2nd passage. Apr. 11. Eye closed; much purulent secretion; vesicles along incisions. Apr. 14. Temperature 41.2°C.; head turns to the right side. Apr. 16. Temperature 40.7°C.; animal circles and falls to the right side. Eyelids open, and inflammation subsiding. Apr. 18. Temperature 39.8°C. Animal still circles and falls. Eye clearer. Apr. 20. Circling is less; animal does not fall. The inflammation of the cornea has subsided and the eye is clear. From this date on improvement continued until recovery was complete.

Comment on this series of experiments will be limited for the present to a few obvious points. It appears, and we had previously observed the fact, that contrary to Doerr's view,[7] fever does attend the eye inflammations, even when there is but moderate brain involvement. But fever and intensity of the ocular inflammation are associated. In order to follow the temperature changes reliably, daily measurements of the rectal temperatures are to be made in all inoculated rabbits. The protocol given shows that recovery may occur from

[7] Doerr, R., *Zentr. Haut- u. Geschlechtskrankh.*, 1924–25, xv, 24.

severe secondary herpetic cerebral inflammation, and the successive inoculations in series indicate that cornea to cornea passages of the H.F. II virus lead to moderation, not to intensification, of its pathogenic action.

Brain and Cord Passages.

In order to compare the mode of action, or virulence, of one strain of herpes virus with another, it is desirable to submit the strains tested to essentially the same treatment. We have observed that a strong, but not a weak, strain of herpes virus when obtained from the brain of a succumbing rabbit is more likely to produce fatal encephalitis on corneal inoculation than the same virus derived from mere corneal passages. Why this difference should arise, is not known; and whether it is merely a quantitative effect, or has to do with a qualitative change in the strain, has not been determined. It will be recalled that the original H.F. I strain of virus, obtained by subdural inoculations of the rabbit, when subsequently instilled into the eyes produced keratoconjunctivitis followed by fatal virus encephalitis in all of the fifteen rabbits inoculated.

The H.F. II virus injected subdurally in rabbits regularly induces stormy symptoms of virus encephalitis and brings about death in from 4 to 6 days. Characteristic histological lesions and inclusion bodies are found on microscopic examination of the brain tissue. But when the infected brain is used for corneal inoculation, a difference appears in that, contrary to the fatal effects of H.F. I virus under these circumstances, recovery ensues from the inflammation induced by H.F. II virus.

Brain to Cornea Passage.—Three protocols are given, in order to show that while the injection of H.F. II vesicular fluid directly into the brain induces fatal virus encephalitis, the brain to cornea passage of the virus which induces severe keratoconjunctivitis sets up only transient brain symptoms.

Rabbit I.—Mar. 30, 1926. 0.1 cc. of salt solution suspension of herpes vesicular fluid injected intracerebrally.[8] Mar. 31. Temperature 41.8°C.; urine reten-

[8] All operations were performed under ether anesthesia.

tion. Apr. 1. Temperature 41.4°C.; tremor. Apr. 3. Tremor increased. Apr. 4. Salivation; gnashing; convulsions; prostration; death.

Rabbit II.—Apr. 6, 1926. Cocainized right eye scarified with cataract knife dipped in salt solution suspension of brain of Rabbit I. Apr. 8. Eye closed, purulent, and vesicles along incisions. Apr. 9. Inflammation severe. Apr. 12. Inflammation subsiding; cornea opaque; circling to right. Apr. 14. Eye clearing; no circling. Recovery was finally complete.

Rabbit III.—Apr. 6, 1926. Right eye treated as with Rabbit II. Apr. 8. Eye closed; severe keratoconjunctivitis; vesicles along incisions. Apr. 8–14. Gradual abatement of inflammation and clearing of the eye. No circling movements seen. Recovery took place.

This experiment may be taken as indicating a difference in quality in two herpes virus strains derived from a supposed virus carrier, that is, a subject of recurrent labial herpes, taken at an interval of 4 years. The next experiment, in which indirect brain, spinal cord, and corneal passages were made, brings out the same general fact, although it shows that the H.F. II virus is capable at times, in passing from the cornea to the brain in sufficient concentration, to set up fatal virus encephalitis.

Brain to Brain to Cornea Passages.—Although the direct passage of the H.F. II virus from brain to cornea failed to produce keratoconjunctivitis followed by encephalitis and death, yet if the virus is passed successively through the brain, it is capable of setting up fatal encephalitis upon corneal inoculation. For example, after the fourth cerebral passage, in which death occurred respectively on the 4th, 6th, 4th, and 4th days, the symptoms having in all instances been characteristic of virus encephalitis, a rabbit was inoculated into the cocainized right eye, the cornea of which had been scarified. The protocol of the test follows.

Rabbit.—May 5, 1926. Eye inoculation with brain virus H.F. II, 4th brain passage, carried on cataract knife used for scarification of cornea. May 7. Eye closed; suppuration; vesicles along incisions. May 9. Circling to right side. May 12. Temperature 41.9°C.; circling; hypersensitive; retention of urine. May 13. Circling; falls to right side; gnashing; salivation. May 14. Convulsions; death.

Indirect Brain, Cord, and Corneal Passage.—The protocols which follow relate to skin to spinal cord, cord and brain to cornea, and cornea to cornea passages.

Rabbit I.—Apr. 13, 1926. Right side of animal shaved and three scratches penetrating into cutis made; a 30 per cent suspension of fresh brain of rabbit of 2nd brain passage rubbed into scratches. Apr. 16. Local inflammation and vesicle formation. Apr. 19. Temperature 40.6°C.; animal moves about slowly. Apr. 21. Paralysis of hind extremities and back; retention of urine. Apr. 22. Fore legs paretic; other symptoms persist. The moribund animal etherized and the pons and lumbar spinal cord employed separately for corneal inoculations.

Rabbit II.—Apr. 22, 1926. Usual corneal inoculation with suspension of pons of preceding animal. Apr. 24. Slight inflammation; no suppuration. Apr. 26. Vesicles along incisions; slight suppuration. Scrapings taken for corneal inoculation of Rabbit IV. Apr. 27. Eye closed; purulent secretion; more pronounced vesiculation. May 1. Tremor. May 2. Temperature 41.2°C.; tremor; convulsions; urine retention. Death occurred on this date.

Rabbit III.—Apr. 22, 1926. Usual corneal inoculation of right eye with suspension of lumbar spinal cord of Rabbit I. Apr. 25. Eye closed; suppuration; vesicle formation. Apr. 27. Circles to right. May 1. Tremor; retention of urine. May 2. Temperature 40.8°C.; convulsions followed by prostration. Death on this date.

Rabbit IV.—Apr. 26, 1926. Right corneal inoculation with scrapings taken from cornea of Rabbit II. Apr. 28. Eye closed; suppuration; vesicle formation. May 1. Circles to right. May 3. Temperature 41.2°C.; tremor; retention of urine. May 5. Convulsions; prostration; death.

With the results of the foregoing experiments before us, we are prepared to place the H.F. II virus among the strains of herpes virus which are dermatotropic, rather than neurotropic in property. This strain of virus is of medium pathogenicity. It tends to produce local inflammation of the cornea and conjunctivæ, and to invade the brain to a slight degree only; and yet, when implanted on the scarified skin and especially when passed successively through the brain, it acquires neurotropic properties of such invasive power as to suffice to induce fatal encephalitis on corneal implantation. Skin inoculation of the virus gives rise to myelitis, with diffusion of the virus throughout the central nervous organs. Although the test is not conclusive on the point, yet the concentration of the virus in the lumbar cord appeared to be greater than in the pons under these circumstances.

A comparison of the activities of H.F. I virus obtained in 1922 with H.F. II virus secured in 1926 from a subject of recurrent labial herpes shows quite conclusively that the pathogenic activity as measured in the rabbit differs. H.F. I virus is predominantly neurotropic, while H.F. II virus is predominantly dermatotropic. Perhaps the isola-

tion and study of a third strain of the virus may throw light on the nature of the variation.

II.

In addition to the tests carried out with the H.F. II strain of herpes virus, two other specimens of virus from subjects of recurrent herpes were studied. One, called T. strain, was derived from a labial vesicle of a female adult suffering from common cold, and the other, called F. strain, from a child of 4 years who passed at intervals of a few months through attacks of slight fever attended by obscure, nervous disturbances slight in degree, and labial herpes. As will appear, the T. and F. viruses stand at extreme scales of pathogenic action, the former being highly and the latter hardly at all neurotropic for the rabbit.

Corneal Passages, T. Strain.

Three passages from cornea to cornea were carried out with the T. strain, as shown in the following protocols. The course of the pathological process was essentially the same in all, consisting of severe keratoconjunctivitis, encephalitis, and death.

Rabbit I.—Sept. 30, 1926. Right eye cocainized and cornea scarified with cataract knife dipped in salt solution containing small quantity of clear exudate of lip herpes vesicle, about 12 hours old. Oct. 2. Inflammation; watery exudate; vesicles along incisions. Oct. 4. Eye closed; purulent exudate. Material taken for inoculation into Rabbit II. Oct. 5–7. Inflammation of eye increased; animal refuses food. Death.

Rabbit II.—Oct. 4, 1926. Usual method of inoculation of right eye after scarification of cocainized cornea with exudate taken on 4th day from Rabbit I. Oct. 6. Eye closed; purulent exudate; vesicle formation. Oct. 8. Temperature 40.6° C.; circles to right. Oct. 9. Circling increased; twists body and falls to right side; gnashing. Oct. 10. Temperature 41.3°C.; convulsions; falls. Oct. 11. Prostration and death.

Rabbit III.—Oct. 11, 1926. Usual method of inoculation of right eye after scarification of cocainized cornea with exudate from eye of Rabbit II. Oct. 15. Temperature 40°C.; eye closed; purulent exudate. Oct. 17. Temperature 41.2°C.; tremor and rapid circling to right side. Oct. 19. Temperature 41.4°C.; hypersensitive; rapid respiration; retention of urine. Oct. 20. Symptoms continue; falls to right and recovers feet with difficulty. Oct. 21. Profuse salivation; convulsions; death.

Brain to Cornea Passages, T. Strain.

A parallel series of inoculations to the former was carried out with the T. virus, in which the original, diluted vesicular contents were inoculated intracerebrally into Rabbit I, and the brain virus thus secured was used to inoculate the cornea of Rabbit II. Rabbit I developed virus encephalitis and succumbed on the 9th day; Rabbit II first developed keratoconjunctivitis, then showed signs of encephalitis on the 6th day, and succumbed to the latter on the 9th day. A third rabbit was inoculated in the cocainized, scarified eye from the brain virus of Rabbit II of the cornea to cornea series. The succession of events was typical: gradual development of keratoconjunctivitis, involvement of the brain, with symptoms of tremor, circling, falling, salivation, and death on the 12th day.

The strain of F. virus was studied in detail, as will appear from the summary of experiments to follow, in the course of which a certain number of disputed points were dealt with and perhaps elucidated.

Cornea to Cornea Passages, F. Strain.

A salt solution suspension of the contents of the labial herpetic vesicle was inoculated by the usual method, that is, by dipping the cataract knife into the suspension and then scarifying the cocainized right cornea of two rabbits with it. Keratoconjunctivitis developed promptly in both animals, and ran the usual course attended by recovery. No symptoms of brain involvement appeared; the highest temperature recorded was 40.7°C., which was reached in both animals.

At the height of the local inflammation (3rd day), exudate was used to inoculate the next two rabbits, constituting the second passage of the series. The events resembled those of the first passage, except that in both animals circling to the right appeared on the 3rd and 4th days respectively. About the 9th day after inoculation the circling abated or ceased, after which recovery was uninterrupted.

In all, ten cornea to cornea passages were made. In no instance was a fatal virus encephalitis induced, and in no case did other signs of brain involvement than that of circling appear. When both eyes were inoculated no circling took place, although the inflammation

produced was typical and severe. Temperatures as high as 41.1°C. arose independently of cerebral symptoms. In the later passages, seventh to tenth, the local reaction was less severe, the temperature lower, and recovery more rapid. Although one eye only was inoculated, no circling occurred.

The conclusions we have drawn from this series of inoculations are:

1. The typical herpes virus keratoconjunctivitis in the rabbit may be attended by fever.

2. Circling to the inoculated side may appear without any other signs of cerebral invasion of the virus.

3. Circling does not appear when the two eyes are inoculated.

4. The virus diminishes in activity in successive corneal passages, as indicated by feebler inflammatory reaction, shorter duration of the inflammation, and absence of circling movements.

Brain and Spinal Cord to Cornea.

The F. virus, as stated, is a weak strain. Tests were carried out in order to determine whether intracerebral injections induced virus encephalitis. It was found as a rule that fatal encephalitis ensued when eye exudate or brain virus was thus injected, but that recovery might occur after intracerebral inoculation.

Rabbit.—Mar. 15, 1926. Animal received intracerebral inoculation of fresh rabbit brain virus. Mar. 19. Temperature 41.1°C.; tremor; retention of urine. These symptoms persisted for 3 days, then abated, and recovery followed.

Experiments were also made in order to determine whether the F. brain virus introduced into the cornea induced both keratoconjunctivitis and frank encephalitis. As a matter of fact, the inflammation of the eye induced is severe, while the effect on the brain is mild only, and if permitted, recedes leaving the rabbit to all intents and purposes normal. And yet if the affected rabbit is killed at the right moment, the virus is detectable by inoculation in the brain tissue.

Rabbits.—Two rabbits, A and B, were given eye inoculations with the fresh F. rabbit brain virus on Sept. 8, 1927. In both, severe keratoconjunctivitis followed. Rabbit A showed fever (40.6°C.) and the head turned to the inoculated (right) side on Sept. 13. On Sept. 15, circling was noted. Killed on this date,

and two rabbits, C and D, were given intracerebral injections of suspension made from the pons.

Rabbit B developed similar symptoms to Rabbit A and was permitted to recover.

Rabbits C and D were given cerebral inoculations with the pons of Rabbit A on Sept. 16. Typical virus encephalitis with characteristic symptoms arose on the 5th and 6th days, and death resulted in both instances on the 9th day.

Since the F. virus is a weak strain, the preceding experiment does not always succeed. In one example, two rabbits were given eye inoculations with material taken from an eye on the 4th day of the keratoconjunctivitis. Severe inflammation attended with circling developed. On the 6th day of the circling, one of the rabbits was killed and the brain used to make a corneal inoculation in one animal, and cerebral inoculation in two animals. No effects followed. At this later period in the encephalitic process, the virus was no longer demonstrable by the inoculation test.

The conclusion to be drawn from these tests is that the F. virus is only weakly neurotropic, and while capable of penetrating to the brain of rabbits, tends to be suppressed there. In this respect, the rabbit treats a weak virus in a manner resembling the way in which the guinea pig also deals with a weak virus.[9] When the quantity of a weak strain reaching the brain of the rabbit is not excessive, it can be destroyed; and yet, this same weak strain when injected in quantity, is capable of producing fatal encephalitis. As is to be expected, a stronger virus, such as the H.F. II strain, is even more readily detected in the pons after eye infection.

Skin to Spinal Cord.

Although the F. virus does not pass from the cornea to the brain in sufficient concentration to produce fatal virus encephalitis, yet it is capable of passing from the skin to the spinal cord in a way to induce paralysis and probably death.

Rabbit.—Mar. 9, 1926. Right side of body shaved, scratched, and covered with 30 per cent suspension of rabbit brain virus taken 5th day after inoculation. Mar. 12. Mild dermatitis. Mar. 15. Dermatitis and formation of vesicles. Temperature 40.9°C. Mar. 17. Curvature of spine; hind legs paralyzed; reten-

[9] Rose, G., and Walthard, B., *Z. Hyg. u. Infectionskrankh.*, 1925–26, cv, 645.

tion of urine. Mar. 18. Temperature 40.8°C.; moves fore legs; almost prostrate; etherized.

Rabbit.—Mar. 10, 1926. Right side of body shaved, scratched, and covered with rabbit brain virus of 6th day. Mar. 13. Dermatitis and vesiculation. Mar. 17–18. Dermatitis subsiding; temperature 40.8°C.; spinal curvature; hind legs weak. Mar. 19–20. Paralysis of back and hind legs complete; tremor; etherized.

Rabbit.—Apr. 8, 1926. Right side of body shaved, scarified, and covered with rabbit brain virus of 7th day. Apr. 11. Dermatitis and vesiculation. Apr. 13. Inflammation subsiding. Apr. 14–15. Temperature 41.3°C.; back muscles and hind legs weak. Apr. 16–21. Complete paralysis of posterior half of body; tremor; etherized.

Immunity.

The experiments with H.F. II virus yielded a number of rabbits in which recovery took place from the corneal inflammation. The animals were subjected to immunity tests in a twofold manner, namely by way of corneal and of intracerebral inoculation. It developed that while the previously uninoculated cornea proved to be but partially protected from infection and inflammation, the brain test showed complete immunity of that more sensitive organ, a result in conformity with observations made by Rose and Walthard.[9] The protocols will be given in pairs, in order to bring out this interesting fact. The brief interval elapsing between the two protection tests is, according to usual measures, insufficient to produce general immunity. As the reinoculations in each set were made on the same day, single control rabbits sufficed for each. Thus it can be stated in advance that the control for the corneal tests developed severe keratoconjunctivitis followed by symptoms of severe virus encephalitis, while the control for the intracerebral tests succumbed to typical virus encephalitis on the 6th day.

Test I. Rabbit.—The corneal test was made 3 months after the first corneal inoculation, and the brain test 12 days after the corneal test. July 19, 1926. Left cocainized, scarified cornea inoculated with 5 per cent suspension of fresh rabbit brain virus. July 22. Eye closed; purulent exudate; small vesicles. July 26. The inflammation gradually subsided. No symptoms of brain involvement. July 29. Eye clear. July 31. Injected intracerebrally 0.1 cc. 5 per cent suspension of fresh rabbit brain virus. No symptoms of any kind followed.

Test II. Rabbit.—In this instance the interval between the two corneal inoculations was 2½ months. July 19, 1926. Left, cocainized, scarified cornea inocu-

lated. July 22. Purulent inflammation and vesicle formation. July 23–27. Eye closed; purulent exudate; then gradual clearing. July 31. Eye clear. Injected intracerebrally with 0.1 cc. of 5 per cent suspension of fresh brain virus. No symptoms of any kind followed.

Test III. Rabbit.—The interval between the first and second corneal inoculations was about 10 weeks. July 19, 1926. Inoculated left, cocainized, scarified cornea. July 22. Eye closed; purulent exudate; vesicles formed. July 28. Eye open and clear. July 31. Intracerebral inoculation of 0.1 cc. 5 per cent suspension of fresh brain virus. No symptoms.

In order to complete the account, it remains to mention the simultaneous tests by intracerebral inoculation of two rabbits which had received corneal inoculations $3\frac{1}{2}$ to $4\frac{1}{2}$ months earlier respectively. The $3\frac{1}{2}$ month animal succumbed to virus encephalitis; the other remained free of symptoms.

Besides the H.F. II animals tested for immunity, five rabbits which had been given corneal inoculations of the F. virus 5 or 6 months before were, along with a control, reinoculated in the left (unused) eye. The point of interest is that while all six rabbits developed keratoconjunctivitis from which they recovered, only the control animal showed the circling symptom.

SUMMARY.

In this paper, three strains of the herpes virus have been dealt with. The H.F. II strain was obtained from the subject H.F. 4 years after the H.F. I strain was secured. H.F. is a victim of recurrent herpes. If the subject is also a chronic carrier of the herpes virus, then it is not one, but two or more strains which are persistently carried. The H.F. II strain is of mitigated pathogenic action for the rabbit, as compared with the H.F. I strain; it is to be classed as dermatotropic rather than neurotropic. And yet, in the subject there was no indication that the attack of herpes provoked was different from the other attacks associated with the H.F. I virus.

The other two herpes strains derive their interest from the fact that they came also from persons who suffer from repeated attacks of labial herpes. One strain proved highly neurotropic, resembling in this respect the H.F. I strain; the other was hardly neurotropic at all, but was none the less definitely dermatotropic. It may be possible

at a later date to secure other samples of virus from these individuals for comparison. The dermatotropic F. strain penetrates to the central nervous system far more readily and certainly from the skin than from corneal surfaces.

The recovered inoculated rabbits showed only relative protection to reinoculation of the herpes virus. A notable difference appeared in the degree of protection acquired, on the one hand by the cornea and on the other by the brain. While the one was partial, the other was complete. The complete resistance of the brain was shown (*a*) by the complete failure of the intracerebral inoculation, and (*b*) by the absence of circling movements following corneal inoculation.

CONCLUSIONS.

Subjects of recurrent labial herpes may yield more than one strain of the herpes virus.

While the H.F. I strain is notably neurotropic, the H.F. II strain, obtained 4 years later, is slightly neurotropic and strongly dermatotropic for rabbits.

The neurotropic property of the H.F. II virus is somewhat increased by brain passage.

Dermal inoculation of the H.F. II strain leads to myelitis, with extension of the virus to the brain. The concentration of the virus in the lumbar cord seems greater than in the pons.

The T. specimen of the herpes virus is apparently of maximal neurotropic potency for rabbits.

The F. specimen of the herpes virus is of low neurotropic and moderate dermatotropic activity. Passage from eye to eye tends to diminish the effect of the virus. When the F. strain is inoculated into one eye, circling occurs; when into both eyes, circling does not occur. None of the corneally inoculated F. rabbits succumbed to virus encephalitis. And yet, the F. virus exists in the brain of the corneally inoculated rabbits and can be detected there, by cerebral inoculation, on the 1st or 2nd day, but not on the 6th day of the circling. When the F. virus does not reach the brain in excessive amounts, it is suppressed there; when injected in large quantity, it induces fatal encephalitis.

The rabbit brain possesses the power of destroying weak strains of the herpes virus in a manner not dissimilar to that possessed by the guinea pig brain.

Immunity tests showed that in rabbits previously inoculated into the cornea, the opposite cornea is only partially, while the brain is wholly, protected against reinoculation effects. The partially protected rabbits developed on corneal reinoculation local lesions, but unlike the control animal, did not show circling movements.

CONTRIBUTIONS TO THE PATHOLOGY OF EXPERIMENTAL VIRUS ENCEPHALITIS.

V. Herpes Virus Encephalitis in the Guinea Pig.

By SIMON FLEXNER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The attempt has been made a number of times to ally epidemic encephalitis in man and herpes virus encephalitis in the rabbit. Ever since Doerr and Vöchting's[1] important observation that rabbits inoculated intraocularly sometimes develop fatal herpes virus encephalitis, this point of view has been in the background. Within a year or two, the view has come more into the foreground of the investigative studies. In two sets of investigations especially, namely those of Levaditi and his coworkers[2] and Doerr and his associates,[3] it has been sought to uphold this view-point. Flexner and Amoss have dealt with Levaditi's contention in another place.[4] It is proposed in the present paper to deal with Doerr's recently published views based on the tests with guinea pigs carried out and described by Rose and Walthard.[5]

As is well known, nothing is easier than to implant the virus contained in herpes vesicles on the rabbit, and to carry the virus in indefinite passages from rabbit to rabbit.

On the other hand, it has proven extremely difficult to implant such a virus on rabbits with material taken from cases of epidemic encephalitis in man. The several hundred or more transfers of these

[1] Doerr, R., and Vöchting, K., *Rev. gén. opht.*, 1920, xxxiv, 409.

[2] Levaditi, C., and Nicolau, S., *Compt. rend. Soc. biol.*, 1924, xc, 1372. Levaditi, C., Nicolau, S., and Poincloux, P., *Compt. rend. Soc. biol.*, 1924, xc, 1376. Doerr, R., *Centr. Bakt., 1. Abt., Orig.*, 1925–26, xcvii, suppl., 76.

[3] Doerr, R., *Centr. Bakt., 1. Abt., Orig.*, 1925–26, xcvii, suppl., 76.

[4] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215.

[5] Rose, G., and Walthard, B., *Z. Hyg. u. Infectionskrankh.*, 1925–26, cv, 645.

materials from man to rabbit have yielded, as Flexner[6] has pointed out and Doerr[3] concedes, six successful inoculations at most. The percentage of successes is almost minimal. The matter at issue is the explanation of the disparity, the burden of proof being of course placed upon those investigators who would identify the herpes virus with the supposedly microbic incitant of epidemic encephalitis.

An explanation is called for all the more emphatically because herpes eruptions carrying virus are so common and ubiquitous an affection of man. The eruptions occur under the most varied circumstances—as attendants of severe disease and as accompaniments of trivial ailments. To ascribe, therefore, to this almost uniformly and universally present—nearly innocent—pathological material the sinister rôle of the inciting microbic agent of epidemic encephalitis is a position not to be lightly assumed or accepted. It does seem rather beside the point to cite, as Doerr[3] has done, as accounting for the irregularity of the infection of rabbits with inocula from cases of epidemic encephalitis, the instance of typhus fever, in which only one-half of the inoculations are successful; or, as has even more recently been urged, the fact that diphtheria bacilli may be carried by persons who have not had and do not acquire diphtheria.[3] The evidences for the existence of a virus of typhus fever are not of this hypothetical order, and those for the specific pathogenic effects of the diphtheria bacillus rest upon a pyramid of knowledge with which the fragile structure of the herpes virus etiology of epidemic encephalitis cannot properly be compared.

The foregoing statement is important only because the undertaking to support the thesis of the herpes virus origin of epidemic encephalitis has recently taken a new turn. It has long been known that while rabbits are highly subject to herpes virus infection, other rodents are far less susceptible to its inoculation. And yet rats, mice, and guinea pigs can be infected, although they often survive even subdural injection of the virus. Recently the guinea pig has been submitted to renewed study under circumstances which have yielded results of a kind to inspire the investigator with new ardor in pursuing the hypothetical relationship between the supposed virus of epidemic encephalitis and the virus of febrile herpes.

[6] Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785. Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215.

As so often happens, two papers dealing with the subject appeared so near together that they may be regarded as having had an almost simultaneous origin. The first was published by Dmitrieff,[7] who states that he chanced on the observation that a strain of herpes virus when introduced into the brain of the guinea pig is quickly reduced in virulence. A study of his protocols shows that even in the first guinea pig transfer this reduction, amounting virtually to destruction, took place. The source of the virus was a brain taken from a rabbit which succumbed to intracerebral inoculation in 3 to 6 days. The rabbit brain material produced encephalitis in young guinea pigs, ending fatally on the 10th or 11th day. When the brains from these guinea pigs were reinoculated into rabbits or other guinea pigs, no obvious effect was produced. When the original rabbit brain virus was injected subdurally into older guinea pigs, either mild symptoms followed by recovery ensued, or no symptoms whatever arose. In one recorded instance the rabbit virus induced keratoconjunctivitis in a guinea pig, and material from the inflamed eye produced, on intracerebral injection, fatal encephalitis in the rabbit.

Although the experiments were few in number, the deductions drawn are far reaching. From the fact that the strain of herpes virus employed was immediately suppressed in the brain of the guinea pig, it is concluded that similar happenings take place in human cases of epidemic encephalitis. In this simple way does Dmitrieff dispose of the innumerable failures to infect rabbits with material taken from cases of epidemic encephalitis. To account for the herpes virus origin of the latter disease, he adopts what is essentially Levaditi's[2] view of special predisposing conditions of the nervous organs coinciding with particularly neurotropic strains of the virus, and he finds in epidemic influenza a possible source of the predisposing agent.

Rose and Walthard's study covered a wider field. So far as their paper is concerned, we need take into account only those parts which bear on our theme, namely the extent to which the reaction of the central nervous system of the guinea pig to the presence of herpes virus may be used as a guide and measure of what goes on in human cases of epidemic encephalitis. The experiments of Rose and Walthard have also, as will appear, a somewhat wider significance, since their inadequacy can be easily traced to the use for the tests of strains of herpes virus not of high but only of moderate virulence. The more deeply the problem of herpes virus is probed, the more clearly is it discerned that original virulence is not only a highly variable quantity,

[7] Dmitrieff, S., *Z. Hyg. u. Infectionskrankh.*, 1926, cvi, 547.

but also that strains are to be selected according to this original grade of virulence for the rabbit, and as suitable for the particular experimental tests which it is proposed to carry out. Had Dmitrieff and Rose and Walthard employed highly virulent strains, their results and conclusions could not but have been very different.

Rose and Walthard sum up their experiments in the following words:

Rabbits inoculated subdurally with the guinea pig brain may succumb to typical encephalitis. Corneal and pad inoculations give poor results. In many instances, however, the subdural inoculations fail. The similarity, therefore, to what happens in von Economo's encephalitis is very close. Should this observation be confirmed, we shall have before us a model, supplied by the use of the guinea pig for inoculation, with which to explain what happens with autopsy material taken from human cases of encephalitis, in which the herpes virus is so rarely demonstrable. While of course this model does not suffice to establish the herpes virus etiology of von Economo's encephalitis, yet the observation does remove one of the most important objections to the assumption of this etiological relationship.

This point of view is upheld by Doerr,[3] in whose laboratory the experiments were made, and by Rose and Walthard. It is, however, based not on many and varied, but only upon a few experiments, of which the following is an instance.

A guinea pig was given a pad inoculation of rabbit brain herpes virus. On the 5th day, paralysis of the extremities appeared, and on the 6th day the animal was killed. The lumbar spinal cord was inoculated into two guinea pigs: in one into the brain and pad, and in the other into the pad and cornea. A control rabbit received a subdural and corneal inoculation. The control developed keratoconjunctivitis and encephalitis, and succumbed on the 5th day.

Now, the guinea pig receiving pad and corneal inoculations responded with both keratoconjunctivitis and paralysis of the hind quarters, from both of which symptoms it recovered. The guinea pig receiving subdural and pad inoculations, responded with paralysis of the hind quarters without showing, however, any signs of encephalitis.

The result is interpreted as showing the extraordinary defense to and capacity for localizing the herpes virus possessed by the central nervous tissues of the guinea pig. A strain of herpes virus capable of passing into and injuring the spinal cord, from a skin surface (so called spontaneous neurotropy),[8] is incapable of attacking the brain on direct injection. Doerr's comment on the experiments is to the effect that many strains of herpes virus are active when inoculated into the skin and cornea, and ineffective when introduced into the brain of guinea pigs. He remarks that this extraordinary variability of the virus is a factor to be reckoned with in considering the etiology of von Economo's encephalitis. In an addendum to his paper, Dmitrieff cites the experiments of Rose and Walthard as upholding his contentions.

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[8] Rose, G., *Centr. Bakt., 1. Abt., Orig.*, 1925–26, xcvii, suppl., 146.

This point of view, that the guinea pig occupies an independent and strategic position in respect to the extraordinary resistance to infection displayed by the brain tissue, is presented by Rose and Walthard from another point of view, namely that of the effects of corneal inoculations in producing symptoms of brain involvement. It will be recalled that Doerr and Vöchting[1] first produced herpes virus keratoconjunctivitis in guinea pigs. Because of the smaller size of the eye, the inflammation is less impressive in guinea pigs. Rose and Walthard are correct in stating that the pronounced opacity of the cornea is a regular symptom of the keratitis in guinea pigs, and only an occasional symptom in rabbits. They state also that they never observed general symptoms (encephalitis) to follow corneal inoculation, although the same strain of virus injected into the pad produces myelitis. They record a single exception to the rule, in which a guinea pig with double corneal inoculation showed salivation for 2 days and later completely recovered from these general effects, as well as from the eye lesions.

The experimental results described by Dmitrieff and by Rose and Walthard are in themselves of interest. The extent of their importance depends, however, upon their general applicability. Unless indeed they are generally applicable, it may be questioned whether their bearing on the disputed question of the etiology of epidemic encephalitis, so called von Economo's encephalitis, is as significant as they suppose. We already know that the highly susceptible rabbit suffices to distinguish differences among strains of herpes virus.[9] May not the less susceptible guinea pig merely act to separate the strains more sharply, by totally suppressing the weak, partly suppressing the medium, and not suppressing at all the strong strains? The experiments which follow would seem to support this interpretation of the reaction of guinea pigs to intracerebral and corneal inoculations of strains of herpes virus.

The experiments to be described have been alluded to in an earlier publication.[10] Moreover, in that publication a protocol is given which shows that a strong virus, implanted on the cornea of the guinea pig, may ascend to the brain and produce fatal encephalitis.

EXPERIMENTAL.

We possess in the H.F. I and Beckley specimens two highly virulent strains of herpes virus. Thus far, these strains have been studied

[9] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 233.
[10] Flexner, S., *J. Gen. Physiol.*, 1925–27, viii, 713.

chiefly in rabbits. Yet as early as 1922, a number of inoculations into guinea pigs were made, and it was found that brain to brain passages took place. Since this is the class of experiment which was carried out more systematically later only sample protocols of the earlier tests will be given here.

Guinea Pigs.—Nov. 20, 1922. Two, 250 gm. guinea pigs, numbered 1 and 2, were inoculated intracerebrally[11] with fresh rabbit brain virus, H.F. I. Guinea Pig 1 showed no striking symptoms, and died on Nov. 26 (brain transferred to Guinea Pigs 3 and 4). Guinea Pig 2 was slow and tremulous on Nov. 27; salivating and convulsive on Nov. 28, on which date death occurred (brain injected into Guinea Pigs 5 and 6).

Nov. 28, 1922. Two, 300 gm. guinea pigs, numbered 3 and 4, received intracerebral injection of brain from Guinea Pig 1. Dec. 1. Guinea Pigs 3 and 4 salivating. Dec. 2. Both guinea pigs convulsive; died on this date.

Nov. 28, 1922. Two guinea pigs, numbered 5 and 6, inoculated intracerebrally with brain from Guinea Pig 2. Dec. 4. Guinea Pig 5 circles to left (side of inoculation) and is tremulous. Guinea Pig 5 died on Dec. 5. Guinea Pig 6 died without showing symptoms.

Further passages were not made. The histology was characteristic of virus encephalitis.

Jan. 23, 1923. Two guinea pigs, 300 gm. each, numbered 9 and 10, received intracerebral injections of fresh rabbit brain, Beckley strain. Guinea Pig 9, Jan. 28, tremulous, ataxic, gnashing. Jan. 29. Salivation. Jan. 30. Death. Guinea Pig 10 circled on Jan. 27 to side of inoculation and died on Jan. 28.

No further passages were made at this time.

Although this series of tests is small and incomplete, it nevertheless brings out two facts: first, that an active virus produces fatal encephalitis in the guinea pig, attended sometimes by symptoms similar to those of the rabbit, and sometimes progressing asymptomatically; and second, that passage of the virus from guinea pig to guinea pig by cerebral inoculation is possible. Note should be given to the demonstration that the brain of an inoculated guinea pig which succumbs without showing symptoms produces on cerebral inoculation of other guinea pigs, encephalitis accompanied by typical symptoms.

Serial Passage of H.F. I Virus.

The repetition of the guinea pig inoculations of the H.F. I virus, undertaken after the papers of Dmitrieff and Rose and Walthard

[11] All operations were performed under ether anesthesia.

appeared, covered a larger number of passages, both by the cerebral and by the corneal routes.

Cerebral Inoculations.

Guinea Pigs.—1st passage. Oct. 25, 1926. Two guinea pigs, A and B, 300 gm. each, inoculated intracerebrally with fresh rabbit brain virus H.F. I. Guinea Pig A developed fever (41.1°C.), tremor, salivation, and weakness of legs; died Nov. 1. Guinea Pig B also developed fever and tremor; died Oct. 29.

2nd passage. Oct. 29. Two guinea pigs, C and D, inoculated intracerebrally with fresh brain, Guinea Pig B. Guinea Pig C developed fever and tremor; died Nov. 4. Guinea Pig D developed, in addition, salivation and convulsions; died Nov. 6.

3rd passage. Nov. 4. Two guinea pigs, E and F, injected intracerebrally with fresh brain of Guinea Pig C. Both showed encephalitic symptoms, including fever (41.2°C.); died Nov. 10 and 12 respectively. The animals which survive longest tend to develop the more striking symptoms.

4th passage. Nov. 12. Two guinea pigs given intracerebrally injection of fresh brain of Guinea Pig E. One died of trauma. The other, Guinea Pig G, showed tremor and salivation; died Nov. 22. Two additional guinea pigs, H and I, were injected intracerebrally with fresh brain of Guinea Pig F on Nov. 10. Both developed symptoms consisting of fever (41.3°C.), tremor, convulsions, and salivation; died Nov. 18 and 19 respectively.

5th passage. Nov. 18. Two guinea pigs, J and K, inoculated intracerebrally with fresh brain of Guinea Pig I. Both developed symptoms of encephalitis and died Nov. 25 and 26 respectively.

6th passage. Nov. 26. Four guinea pigs were inoculated, two with fresh brain of Guinea Pig J, and two with fresh brain of Guinea Pig K. One of the four died of trauma; the other three (Guinea Pigs L, M, and N) showed typical symptoms of encephalitis; died on Dec. 4 and 5.

7th passage. Dec. 6. Four guinea pigs were injected intracerebrally with fresh brain of Guinea Pigs L and M of previous passage. All (Guinea Pigs O, P, Q, R) developed characteristic symptoms; died Dec. 12 and 13.

8th passage. Dec. 14. Two guinea pigs (S and T) inoculated intracerebrally with fresh brain of Guinea Pig R, 7th passage. Both developed symptoms; died Dec. 20.

9th passage. Dec. 21. Two guinea pigs (U and V) injected intracerebrally from Guinea Pig S of 8th passage. Both developed symptoms. One (U) died Dec. 27; the other (V) recovered.

10th passage. Dec. 28. Two guinea pigs (W and X) inoculated intracerebrally with fresh brain of Guinea Pig U, 9th passage. Both guinea pigs developed symptoms; died Jan. 3, 1927.

At this point, the series of inoculations was interrupted. The results of the series were established by histological study of the brains of the guinea pigs and by return inoculations intracerebrally and corneally into rabbits at the second and third guinea pig passages. Five rabbits receiving inoculations of guinea pig brains, either subdurally or corneally, developed encephalitis and succumbed. The fact should be stressed that virus encephalitis in the guinea pig sometimes runs an asymptomatic course to a fatal issue. The brains of such animals produce symptomatic encephalitis on passage inoculation. This condition was observed in the 1922 and again in the 1926 virus passages.

Corneal Inoculations.

The series of corneal inoculations of guinea pigs with H.F. I virus assumes importance by reason of the fact that Rose and Walthard[5] and Doerr[3] emphasize the point that no strain of herpes virus has been described which passes from the eye to the brain, inducing fatal encephalitis. The H.F. I virus is capable of making this passage and of causing death.

Guinea Pigs.—Guinea Pig I. Nov. 4, 1926. Right eye cocainized and cornea scarified with cataract knife dipped in 10 per cent suspension of fresh brain of Guinea Pig C, 2nd cerebral passage. Severe keratoconjunctivitis followed, attended by fever (41.2°C.), and on 6th day by symptoms of brain involvement: circling to right, tremor, ataxia, salivation. Death on Nov. 16, or 12 days after inoculation.

Guinea Pig II.[12] Nov. 8. Right eye inoculation as before with exudate from Guinea Pig I. First keratoconjunctivitis, then encephalitis developed, leading to death on Nov. 18, or 10th day after inoculation.

Further inoculations were made with the fresh brains of Guinea Pigs I and II. Material from Guinea Pig I was introduced into the cornea of one guinea pig and the brain of two other guinea pigs, without effect. The conclusion is that the virus was no longer active on the 12th day. The brain of Guinea Pig II was injected intracerebrally into two guinea pigs, of which one succumbed to virus encephalitis on the 6th, and the other on the 12th day. Another guinea pig which received a corneal inoculation of the brain, developed keratoconjunctivitis attended by salivation, from which recovery took place.

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[12] This is the animal whose protocol is given in "Epidemic encephalitis and simple herpes," Flexner, S., *J. Gen. Physiol.*, 1925–27, viii, 713.

The power to pass from the cornea to the brain possessed by even strong strains of herpes virus is determined not by the virus alone, but is conditioned by the individual guinea pig subjected to inoculation. Thus of four guinea pigs which were given corneal inoculations of fresh H.F. I guinea pig brain virus, one at the third and three at the sixth guinea pig passage, all four developed keratoconjunctivitis, but one only exhibited encephalitic symptoms to which it succumbed. The protocol of this animal follows.

Guinea Pig.—Dec. 4, 1926. Cocainized cornea scarified and inoculated with H.F. I virus, 6th guinea pig brain passage. Typical keratoconjunctivitis. Dec. 11. Salivation, tremor, falling. Dec. 13. Death.

There remains to be described one other type of corneal inoculation with strong herpes virus, namely that in which following the keratoconjunctivitis the guinea pigs develop encephalitic symptoms from which they recover. The next two protocols illustrate this result.

Guinea Pig.—Oct. 25, 1926. Right eye cocainized and scarified with cataract knife dipped in suspension of fresh rabbit brain virus H.F. I. Typical keratoconjunctivitis appeared. On 6th day the temperature rose to 41.2°C.; on 10th day, tremor, ataxia, and circling to right were noticed. These symptoms and the inflammation of the eye gradually subsided, recovery becoming complete.

Guinea Pig.—Oct. 29, 1926. Right eye inoculation as before with fresh guinea pig brain virus H.F. I, 2nd brain passage. Moderately severe keratoconjunctivitis. On 10th day, tremor and salivation, and temperature of 40.7°C. During the next few days the eye inflammation subsided and the nervous symptoms disappeared.

We shall now describe the inoculation of guinea pigs with the Beckley and Levaditi strains of herpes virus. These two strains have been studied by us in rabbit passages and found to be weaker than H.F. I.[9] Moreover, they differ from each other, as the Levaditi strain is weaker than the Beckley. As tested on the rabbit, the order of virulence is H.F. I, Beckley, and Levaditi.

Beckley Virus Series.

Two separate tests were made with the Beckley virus injected intracerebrally into guinea pigs. The two sets are not in precise agreement, since the fatalities in one exceeded those in the other test. But they are in agreement in bringing out the fact that the Beckley is less active than the H.F. I strain.

Cerebral Inoculations.—

Test I. Guinea Pigs A and B.—Dec. 31, 1926. Injected into right cerebrum suspension of fresh rabbit brain Beckley virus. Fever (41.1°C.), tremor, salivation, and paralysis of extremities developed. Death of Guinea Pig A occurred on Jan. 8, 1927. Guinea Pig B was killed on Jan. 6.

Guinea Pigs C and D.—Jan. 7, 1927. Inoculated as in preceding test with fresh brain virus of Guinea Pig B. Symptoms of encephalitis appeared in both guinea pigs and death followed on Jan. 14.

Guinea Pigs E and F.—Jan. 17, 1927. Injected as in preceding, with fresh brain virus of Guinea Pig D. Symptoms of encephalitis developed and Guinea Pig E died on Jan. 24; Guinea Pig F died on Jan. 22.

Guinea Pig G.—Jan. 25, 1927. Inoculated as before with fresh brain virus of Guinea Pig E. Developed symptoms of encephalitis, including tremor, gnashing, convulsions, salivation, and impaired eyesight; death on Feb. 2.

Guinea Pig H.—Feb. 4, 1927. Inoculated as before with fresh brain virus of Guinea Pig G. No symptoms appeared and animal remained well.

Guinea Pigs I and J.—Feb. 7, 1927. Injected intracerebrally with virus from Guinea Pig G, glycerolated for 5 days. No effect was produced.

The sudden loss of activity on the part of the Beckley strain of virus at the fifth passage in guinea pigs led us to repeat the test. The series of protocols follows.

Test II. Guinea Pigs A and B.—Feb. 21, 1927. Intracerebral inoculation of fresh rabbit virus, Beckley strain. Symptoms of severe encephalitis developed, so that the moribund animals were killed on Feb. 28 and Mar. 3 respectively.

Guinea Pigs C and D.—Mar. 1, 1927. Cerebral inoculation from Guinea Pig B. Symptoms of encephalitis, including tremor, salivation, and convulsions, appeared. Guinea Pig C died on Mar. 7; Guinea Pig D was killed on Mar. 5.

Guinea Pigs E and F.—Mar. 5, 1927. Cerebral inoculations from Guinea Pig D. Guinea Pig E showed slight, fleeting symptoms only and recovered; Guinea Pig F showed more pronounced, but not severe symptoms, and was killed on Mar. 11.

Guinea Pigs G and H.—Mar. 11, 1927. Cerebral inoculation with fresh brain of Guinea Pig F. No marked symptoms arose and animals remained well.

The result of the passage of the Beckley virus through the brains of guinea pigs is definite and shows that the virus, as derived from the rabbit, is active enough in the first removes to produce fatal encephalitis, but at the fourth or fifth passage suddenly loses power and fails to excite marked symptoms in the inoculated guinea pigs.

Corneal Inoculations.—The preceding tests were paralleled with two series of tests of the Beckley virus inoculated into the cornea. The first of the eye series is incomplete, but is recorded for its intrinsic

interest. The second is complete and brings out the rather sudden failure in potency of the virus in the manner of the intracerebral passages.

Test I. Guinea Pigs A and B.—Dec. 30, 1926. Cocainized right eye scarified with cataract knife dipped in 10 per cent suspension of fresh rabbit brain Beckley virus. Characteristic keratoconjunctivitis followed by fever (41.1° to 41.7°C.) on Jan. 6, 1927, coincident with appearance of tremor, salivation, and convulsions. Death of both animals on Jan. 10.

Guinea Pigs C and D.—Jan. 11, 1927. Intracerebral injections of fresh brain virus of Guinea Pig A. Typical symptoms of encephalitis developed; death on Jan. 18 and 19 respectively.

Guinea Pigs E and F.—Jan. 10, 1927. Corneal inoculations with fresh brain of Guinea Pig A. Keratoconjunctivitis followed by encephalitis appeared; death of Guinea Pig E on Jan. 17, of Guinea Pig F on Feb. 4.

Test II. Guinea Pigs A and B.—Jan. 6, 1927. Corneal inoculations with fresh brain of guinea pig of 1st Beckley strain passage. Keratoconjunctivitis and encephalitis; death on Jan. 17 and 21 respectively.

Guinea Pigs C and D.—Jan. 14, 1927. Corneal inoculation with fresh brain of guinea pig of 2nd Beckley strain passage. Keratoconjunctivitis and encephalitis; death of both animals on Jan. 29.

Guinea Pigs E and F.—Jan. 24, 1927. Corneal inoculation with fresh brain of guinea pig of 3rd Beckley strain passage. Keratoconjunctivitis and encephalitis; death of Guinea Pig E on Feb. 4, and sacrifice of F on Feb. 2.

Guinea Pigs G and H.—Feb. 2, 1927. Corneal inoculation with fresh brain of guinea pig of 4th Beckley strain passage. Severe keratoconjunctivitis without symptoms of encephalitis followed by recovery.

Guinea Pigs I and J.—Mar. 11, 1927. Corneal inoculation with fresh brain of guinea pig of 3rd Beckley strain passage. The cornea of one, but not of the other guinea pig became inflamed, and no cerebral symptoms appeared.

Levaditi Virus Series.

The experiments so far described establish the guinea pig as selective for strains of herpes virus of differing potencies as is the rabbit, the distinction being that because of greater natural resistance to infection the guinea pigs make a sharper distinction of virulence than do rabbits. Hence we should expect that a strain of the virus relatively weak for the rabbit should prove even less effective for the guinea pig than either the H.F. I or the Beckley strain. Levaditi Strain 6 is, according to Flexner and Amoss,[9] such a weak strain. It has, therefore, been inoculated into guinea pigs by way of the brain and the cornea, and has

behaved in conformity with its rabbit propensities. Of the three strains studied by us in guinea pigs, it alone corresponds in its action to the descriptions given by Dmitrieff and by Rose and Walthard. The protocols of the experiments follow in brief.

Test I. Guinea Pigs I and II.—Jan. 26, 1927. Cerebral inoculation with fresh rabbit brain Levaditi virus. Guinea Pig I showed no symptoms and remained well. Guinea Pig II developed fever (40.8°C.) and tremor on the 3rd day. No accentuation of the symptoms but emaciation set in. Killed on 13th day. The brain of this animal injected intracerebrally into two guinea pigs, III and IV, and into a rabbit was without result.

The original rabbit brain virus inoculated into the cornea of two guinea pigs produced keratoconjunctivitis, unattended by cerebral symptoms from which recovery ensued.

Test II. Guinea Pigs V and VI.—Feb. 15, 1927. Cerebral injection of fresh rabbit brain Levaditi virus. No symptoms followed and the animals remained well.

Test III. Two Guinea Pigs.—Feb. 22, 1927. Injected as in previous tests with fresh rabbit brain Levaditi virus without producing symptoms.

Test IV. Two Guinea Pigs.—Mar. 23, 1927. Received fresh rabbit brain Levaditi virus by cerebral injection, again without result.

The Levaditi virus employed for the inoculations was active for the rabbit on intracerebral injection and was used on the 6th day of the encephalitis for injecting the guinea pigs.

As the protocols show, only the first cerebral inoculation was positive and that in the first test only. In this test, the attempted second passage to guinea pigs failed. Corneal inoculation succeeded in the first test, but no evidence of extension of the inflammatory process to the brain was detected.

DISCUSSION.

The experiments described in this paper leave no doubt that the guinea pig is only less subject to infection with the herpes virus than is the rabbit. The inoculations of herpes virus in the guinea pig in the past have covered too narrow and restricted a variety of virus strains. There is no doubt that the strain employed by Dmitrieff was a weak one, and the one employed by Rose and Walthard little, if any, stronger. Flexner and Amoss[9] had already placed the Doerr strain—presumably the one used by Rose and Walthard—among the weak strains, on the basis of rabbit tests and of reaction to glycerolation.

The Levaditi strain had also been classed as a weak strain; and the tests on guinea pigs bring these three strains—Dmitrieff, Rose and Walthard, and Levaditi—in the same category of strength or virulence. The fact should be emphasized that so weak a virus as the Doerr strain, inoculated into the skin of guinea pigs, proved to be active enough to pass regularly from it to the spinal cord, thus displaying the property called spontaneous neurotropy by Rose.[8]

The H.F. I and Beckley strains of virus possess far greater virulence for guinea pigs as well as for rabbits. Their strength has not notably diminished during the 5 and 4 years respectively since their original isolation and after very many rabbit passages. Both are capable of being passed from guinea pig to guinea pig by means of cerebral and corneal inoculations, although the H.F. I strain, the stronger by rabbit test, alone seems capable of indefinite passage. Both also are capable of passing from the cornea to the brain, inducing fatal encephalitis. Hence they belong in a totally different category from the three other strains already mentioned.

The theoretical importance of the manner of response of guinea pigs to herpes virus inoculation arises from the use which has been made of fragmentary knowledge to support the hypothesis of the virus etiology of epidemic encephalitis. The paradox of this ubiquitous virus, ordinarily so readily implanted upon the rabbit and yet so rarely found in cases of epidemic encephalitis, has effectually barred the way to the wider adoption of the hypothesis. Hence the eagerness with which its upholders seized upon the guinea pig experiments as supporting their belief. The experiments given in this paper make it more than doubtful whether the support is valid and whether, indeed, any light whatever has been thrown on the etiology of epidemic encephalitis through the tests made on guinea pigs. Rather it would seem that the nature of the inciting agent of that disease remains still a problem for future solution.

SUMMARY.

The guinea pig is subject to cerebral and corneal inoculation of the herpes virus.

The effects of the inoculations vary with the strength or degree of virulence of the virus.

Weak strains of the virus are implanted on the cerebrum with difficulty and strong strains with ease.

Weak strains are quickly suppressed by the brain and strong strains may be passed indefinitely from brain to brain of the guinea pig. Strains of intermediate potency can be passed for a limited number of times only.

Weak strains induce keratoconjunctivitis without brain involvement, while strong strains invade the brain from the eye and produce fatal encephalitis. In the latter case, the brain contains active virus inoculable upon the cornea and into the brain of rabbits and guinea pigs. Strains of intermediate potency produce keratoconjunctivitis accompanied by mild symptoms of encephalitis, from which recovery results.

The guinea pig serves even more definitely than the rabbit to distinguish grades of virus according to strength or virulence. There is no difference of kind but only of degree of response to inoculation of herpes virus in the rabbit and the guinea pig.

The etiology of epidemic encephalitis has not, therefore, been brought appreciably nearer solution by experiments with herpes virus carried out in guinea pigs.

I wish to thank Mr. Peter Haselbauer for very valuable assistance in the carrying out of the experiments on which the two present papers are based.

ULTRA-VIOLET LIGHT AND VACCINE VIRUS.

I. The Reaction of Irradiated Skin to Vaccine Virus.

By THOMAS M. RIVERS, M.D., HENRY STEVENS, and FREDERICK L. GATES, M.D.

(*From the Hospital and the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 5.

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Ledingham (1) reported that the injection of India ink into the skin of rabbits induced a local refractory state to the action of vaccine virus, *i.e.*, the visible reaction usually produced by vaccine virus failed to occur in parts of the skin previously treated with ink. Carnot and his coworkers (2) determined that areas of rabbits' skin repeatedly exposed to ultra-violet light were less susceptible to the action of vaccine virus than were untreated areas of skin in the same animal. Le Fèvre de Arric observed that the shaved skin (rabbits') exposed to Roentgen rays was more resistant 14 days later to vaccine virus than was untreated skin (3). The observations of these men and a desire to obtain information concerning the modification of the picture of vaccinia in rabbits by means of non-specific procedures led to the experiments which are reported in the present paper.

Methods and Materials.

Vaccine Virus.—The vaccine virus used in these experiments was obtained originally from Dr. Noguchi. It is free from ordinary bacteria and is propagated in the testicles of rabbits (4). A large amount of the virus was prepared and glycerolated. To facilitate comparison of results, the same virus emulsion was used in all experiments.

Animals.—The majority of the rabbits used were albinos. Other kinds of rabbits were employed, however, and the results observed in them were similar to those obtained in albinos.

Irradiation and Vaccination.—The hair was removed from large areas of skin by shaving. In the animals that received only one irradiation, selected areas of

the shaved skin were exposed to the direct rays of the quartz mercury arc (60 to 65 volts D. C.) at a distance of 50 cm. for periods of time ranging from 5 to 40 minutes. The control areas of skin were protected against the action of the ultra-violet light by screens of lead-foil. At different intervals of time following the completion of the irradiation the treated and untreated areas of skin were scarified and inoculated with vaccine virus. In the rabbits that received repeated irradiations, shaved areas of skin over the dorsal surface of the animals were exposed to the action of ultra-violet light at a distance of 1 meter for 15 to 30 minutes, at daily intervals for 1 or 2 weeks. After the repeated exposures to the light, the treated skin was scarified and inoculated with vaccine virus. Control areas of skin of the same animals, on the lateral surface of the abdomen and thorax, were shaved and inoculated in a manner similar to that employed in the treated areas. As an additional control, the shaved skin over the dorsal surface of rabbits not treated with ultra-violet light was also scarified and inoculated with vaccine virus.

In studying the effect that ultra-violet light has upon the skin of rabbits in regard to the activity of vaccine virus subsequently inoculated upon it, the work was divided into two parts; the first dealt with the effect of single exposures, the second with repeated irradiations.

Effect of Single Irradiations.

A number of rabbits were exposed to the rays of the quartz mercury arc for 5, 10, or 30 minutes. 30 to 60 minutes after the exposures to the light were completed, areas of treated and untreated skin were scarified and inoculated in the usual manner with vaccine virus. Nine experiments were performed in the first series. The results are summarized in Table I.

The results shown in Table I indicate that the activity of vaccine virus is delayed and mitigated (see Fig. 1) when the virus is inoculated on the recently irradiated skin of a rabbit. Furthermore, the lesions that develop in treated skin are frequently more deeply situated than are those in untreated skin.

The fact that the lesions in the irradiated skin were delayed and more deeply situated than usual led us to suspect that they might have resulted from virus transported to the treated skin through the blood stream from the lesions in the untreated skin. Experiments were performed to obtain information concerning the source of the virus producing these deep lesions.

An area of a rabbit's skin was irradiated for 40 minutes in the usual manner. Thirty minutes later the irradiated skin was gently scarified and inoculated with vaccine virus. Care was taken not to soil the untreated skin with virus. Another rabbit whose skin had not been exposed to ultra-violet light was inoculated in the usual manner. The lesions in the control rabbit appeared on the 3rd day, and

TABLE I.

The Reaction of Irradiated Skin to Vaccine Virus Inoculated upon It Shortly after Single Irradiations.

Rabbit No.	Distance from arc	Time of exposure	Voltage of D.C.	Results of vaccination	
				Irradiated skin	Untreated skin
	cm.	*min.*			
1	50	30	60–65	One pustule appeared 6th day	Eleven pustules appeared 3rd day
2	"	"	"	No lesions	Numerous lesions
3	"	"	"	+	+++
				First definite sign 6th day	Appeared 3rd day
4	"	"	"	+	++++
				First definite sign 6th day	Appeared 3rd day
5	"	"	"	+	++++
				First definite sign 6th day	Appeared 3rd day
6	"	"	"	?	++
				Doubtful take 6th day	Appeared 3rd day
7	"	"	"	?	+++
				No definite lesions	Appeared 3rd day
8	"	5	"	++	+++
				Appeared 4th day	Appeared 3rd day
	"	10	"	+	+++
				Appeared 4th day	Appeared 3rd day
9	"	5	"	++	+++
				Appeared 4th day	Appeared 3rd day
	"	10	"	+	+++
				Appeared 4th day	Appeared 3rd day

+ indicates a positive reaction to vaccine virus. The number of + signs indicates the severity of the reaction.

? indicates the presence of a doubtful reaction.

were confluent. Only a few lesions developed in the treated rabbit; they appeared on the 5th day and were deeply situated in the skin. Several experiments of this character were performed and the results were always the same, provided a potent virus was used.

Two rabbits were shaved on both sides of the thorax and abdomen. Two areas on one side of each rabbit were irradiated for 20 and 40 minutes respectively.

Strips of skin for controls were protected from the effect of the light. Immediately after irradiation the untreated side of each rabbit was inoculated with vaccine virus in the usual manner. The lesions at the sites of inoculation appeared on the 3rd day, and later coalesced. On the 5th day a few discrete lesions were observed in the irradiated skin. None were seen, however, in the nearby skin protected by foil.

The results of the two sets of experiments just described indicate that the deep, delayed lesions in the irradiated skin of the rabbits employed for the experiments summarized in Table I may have resulted either from the virus inoculated directly on the treated skin or from virus transported to it by the blood from lesions in the untreated skin.

It seemed of interest to ascertain whether irradiated skin possesses virucidal properties for vaccine virus. This matter was investigated in the following manner.

The skin on one side of a rabbit was irradiated for 40 minutes. Immediately after the completion of the irradiation the rabbit was sacrificed. The superficial layers of the treated skin were then removed and emulsified. As a control, skin from the untreated side was removed and emulsified in a similar manner. Portions of each emulsion were mixed with small amounts of vaccine virus and incubated at 37°C. for 1 hour. These mixtures were then smeared on areas of scarified skin of the same rabbit. Lesions developed at all points where the mixtures were placed.

From the above experiment it may be concluded that irradiated skin is not virucidal for vaccine virus and that the results summarized in Table I were not dependent upon a factor of this nature.

When it had been determined that the reaction to vaccine virus was delayed and mitigated in irradiated skin inoculated 30 to 60 minutes after treatment, experiments were performed to ascertain the type of reaction that occurs in skin inoculated with vaccine virus 18 to 72 hours after irradiation.

A number of rabbits were shaved and areas of skin were irradiated for 5, 10, or 30 minutes. Areas of skin for control on each rabbit were protected by foil. Areas of treated and untreated skin were inoculated with vaccine virus after varying intervals of time had elapsed following irradiation. At the time of inoculation the treated skin was usually red and edematous. The results of the experiments are summarized in Table II.

From the results of experiments summarized in Table II it appears that skin irradiated for a short time 24 to 72 hours prior to inoculation

TABLE II.

The Reaction of Irradiated Skin to Vaccine Virus Inoculated upon It 18 to 72 Hours after Single Irradiations.

Rabbit No.	Distance from arc	Time of exposure	Voltage of D.C.	Lapse of time between irradiation and inoculation	Results of vaccination	
					Irradiated skin	Untreated skin
	cm.	*min.*		*hrs.*		
10	50	30	60–65	24	+++ Appeared 4th day	+ Appeared 4th day
11	"	"	"	"	+++++ Appeared 3rd day*	+++ Appeared 2nd day
12	"	"	"	"	++++ Appeared 3rd day	+++ Appeared 2nd day
13	"	"	"	"	++++ Appeared 3rd day Slow in developing	+++ Appeared 2nd day
14	"	"	"	18	± Interpretation difficult	+++ Appeared 3rd day
15	"	"	"	"	± Interpretation difficult	+++ Appeared 3rd day
16	"	5	"	48	+++++ Appeared 3rd day	++++ Appeared 3rd day
	"	10	"	"	+++++ Appeared 3rd day	++++ Appeared 3rd day
17	"	5	"	72	+++++ Appeared 2nd day	++++ Appeared 2nd day
	"	10	"	"	+++++ Appeared 2nd day	++++ Appeared 2nd day

* Frequently difficult to determine the time of appearance of the lesions in the irradiated skin, inasmuch as the skin was red and edematous as a result of the irradiation.

+ indicates a positive reaction to vaccine virus. The number of + signs indicates the severity of the reaction.

± indicates a positive reaction masked by the irritation of the skin produced by the light.

reacts more violently to vaccine virus than does untreated skin in the same animal (see Fig. 2).

Effect of Repeated Irradiations.

The effect of single exposures to ultra-violet light upon the types of reaction produced by vaccine virus subsequently inoculated on the treated skin aroused interest in the question as to what effect repeated irradiations would have upon such reactions. The matter was investigated in the following manner.

A number of rabbits were shaved. Certain areas of skin were irradiated for 15 or 30 minutes daily for about a fortnight. At first the skin became red after each irradiation, and after a few treatments desquamation of the superficial layers of the epidermis occurred. Subsequent to this no macroscopic changes were noted in the skin, following the daily irradiations. Finally the treated skin was inoculated with vaccine virus 1 hour or 48 hours after the last exposure. Areas of untreated skin in the same animals and the skin of untreated rabbits were also inoculated with virus as controls. At the time of inoculation the irradiated skin appeared to be perfectly normal. The results of the experiments are shown in Table III.

TABLE III.

Effect of Repeated Irradiation upon the Skin in Respect to Its Reaction to Vaccine Virus.

Rabbit No.	Distance from arc	Voltage of D.C.	Time of each exposure	No. of exposures	Lapse of time between last irradiation and inoculation	Results of vaccination		
						Irradiated skin	Untreated skin	Skin of untreated animal
	m.		*min.*		*hrs.*			
18	1	60–65	30	9 in 11 days	1	++	No test	++++
19	"	"	"	11 " 13 "	"	+	+++	++++
20	"	"	15	12 " 14 "	"	++	++++	No test
21	"	"	"	11 " 12 "	48	±	++++	" "

+ indicates a positive reaction to vaccine virus.

± indicates a weakly positive reaction to vaccine virus. The number of + signs indicates the severity of the reaction.

From the results of the experiments summarized in Table III, one may conclude that areas of skin repeatedly irradiated are less susceptible to the action of vaccine virus than are untreated areas of skin in the same animals.

DISCUSSION.

Rabbits' skin irradiated for a few minutes and then immediately inoculated with vaccine virus is less susceptible to the action of the virus than is untreated skin. If 24, 48, or 72 hours elapse between the time of irradiation and inoculation, however, the treated skin appears to be more susceptible than untreated skin. Teague and Goodpasture (5) have demonstrated that herpes virus produces an especially pronounced reaction in a guinea pig's skin that shows active epithelial hyperplasia produced by applications of coal tar. Cobbett and Melsome (6), working with hemolytic streptococci, noted that rabbits' ears irritated with mustard oil were more susceptible to streptococcal infections than were control ears during the first 48 hours following the irritation, after which they became more resistant than the controls,—a finding the converse of that in our irradiated rabbits.

An explanation of these phenomena and of that which we have dealt is not readily found. Certain filtrable viruses (7), among which are vaccine virus and herpes virus, have not been cultivated in lifeless media. These active agents, however, do multiply in the presence of suitable living host cells. Furthermore, the multiplication seems to occur best in the presence of young, actively growing cells. These facts may aid in explaining the results of some of the experiments reported in the present paper. When irradiated skin was immediately inoculated with vaccine virus, the virus was placed on damaged or dead tissue,—for in the end the skin desquamated. One may presume that the reaction to the virus was delayed or mitigated because of the condition of the cells with which it came in contact. If 24, 48, or 72 hours elapsed between the time of irradiation and inoculation, the virus was placed on a skin the surviving cells of which were already stimulated toward repair while multitudes of others had come in from the blood stream. Falling on such a soil the vaccine virus may well have acted more intensely than usual. This explanation is suggested merely as a possibility.

The skin that was repeatedly exposed to ultra-violet light appeared to be normal except for a light tan noted in a few instances. Yet in spite of its normal appearance, this irradiated skin was less susceptible to the action of vaccine virus than was untreated skin. No reason can at present be offered for the enhanced resistance to vaccine virus.

It is obvious that the reaction of the skin to certain viruses may be modified by means of non-specific procedures. Furthermore, since a great deal of the evidence in favor of the identity of some of the epitheliotropic viruses has been obtained by means of cross-immunity experiments conducted in the skin of human beings and animals, it is possible that factors of a non-specific nature, generally overlooked or underestimated, are responsible for part of the confusion concerning the interrelationship existing between certain of these viruses.

SUMMARY.

Rabbit skin treated for a few minutes with ultra-violet light and then inoculated at once with vaccine virus is less susceptible to the action of the virus than is untreated skin. If 24, 48, or 72 hours elapse between the time of irradiation and inoculation, the treated skin appears to be more susceptible than is untreated skin. Skin repeatedly exposed to ultra-violet light is less susceptible to the action of vaccine virus than is non-irradiated skin.

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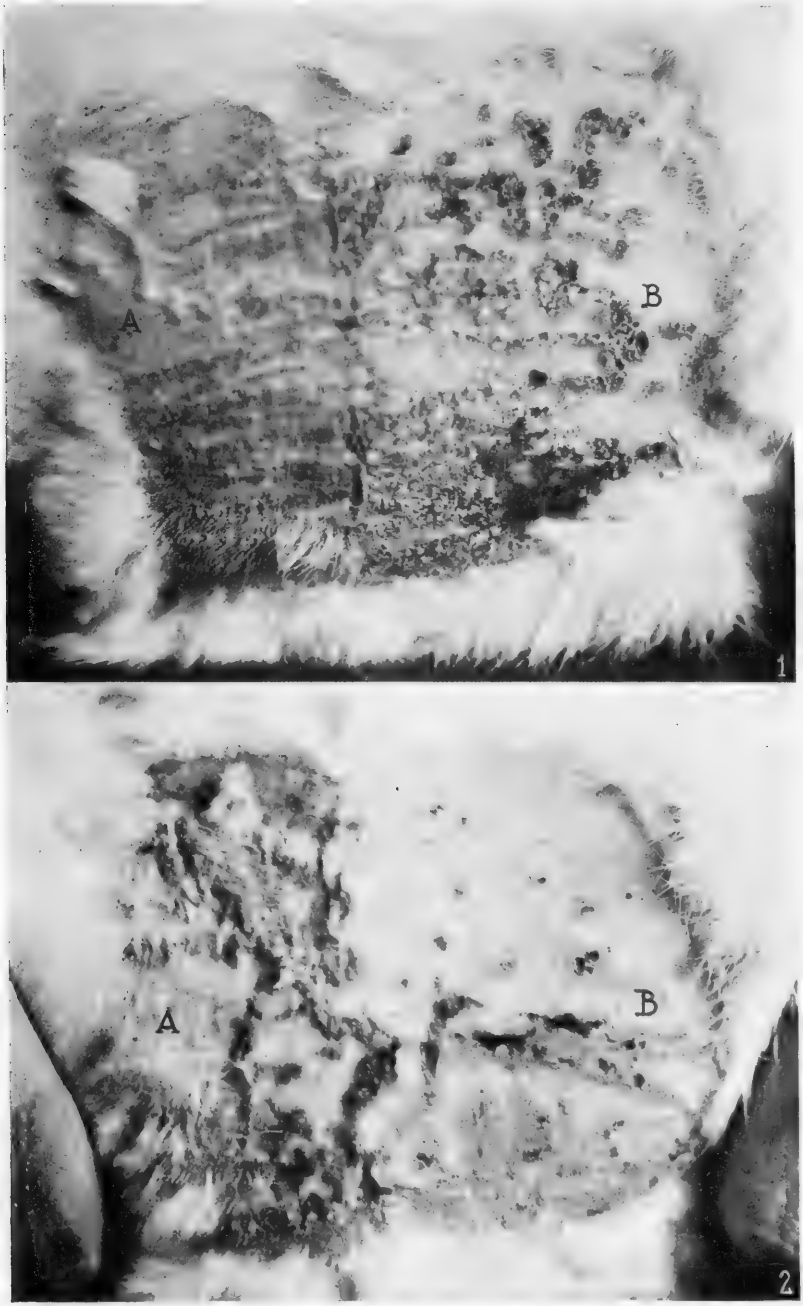
EXPLANATION OF PLATE 5.

FIG. 1. Rabbit 1961. *A* irradiated, *B* control. Both *A* and *B* were inoculated with vaccine virus within a few minutes after irradiation of *A*. The broad dark lines observed in *A* are the effects of scarification and do not represent a reaction to vaccine virus.

FIG. 2. Rabbit 1960. *A* irradiated, *B* control. Both *A* and *B* were inoculated with vaccine virus 24 hours after irradiation of *A*.



(Rivers, Stevens, and Gates: Ultra-violet light and vaccine virus. I.)

ULTRA-VIOLET LIGHT AND VACCINE VIRUS.

II. The Effect of Monochromatic Ultra-Violet Light upon Vaccine Virus.

By THOMAS M. RIVERS, M.D., and FREDERICK L. GATES, M.D.

(From the Hospital and the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 28, 1927.)

It has been known for many years that ultra-violet light is bactericidal. Finsen and Dreyer (1), in 1903, were probably the first to show that light with short wave-lengths is virucidal also. More recently (2–7) other reports have appeared dealing with the effects of ultra-violet irradiation upon bacteria, viruses, and enzymes. For the most part, however, single wave-lengths have not been used in the investigations, nor have the workers determined or taken into consideration the amount of energy required to kill or to inactivate these agents at single wave-lengths in the active ultra-violet region. One of the present authors (Gates) is making a quantitative study of the action of ultra-violet light on certain active agents in the hope of obtaining additional information concerning their nature. In the present paper the results of the studies concerning the effects of ultra-violet irradiation upon one of these active agents, vaccine virus, will be presented.

Methods and Materials.

Vaccine Virus.—The source and preparation of the vaccine virus have been described in the preceding paper. Fresh virus, however, instead of glycerolated material, was used in the experiments to be reported in the present paper.

Method of Irradiating and Testing the Virus.—Three drops of fresh virus were allowed to spread over the surface of 2 per cent nutrient agar (pH 7.4) in a small Petri dish. After being surface-dried in a horizontal position, certain areas of these plates were exposed for various intervals of time before the exit slit of a large quartz monochromatic illuminator to the different spectral lines of a quartz mercury arc in the ultra-violet region between λ 2302 and λ 3126 Ångström units. The intensity at each wave-length (measured in ergs per sq. mm. by means of a

standardized thermopile and high sensitivity galvanometer) multiplied by the time of exposure gave the total incident energy for each exposure. For comparison, a thin suspension of *Staphylococcus aureus* was washed over the surface of nutrient agar also. After surface drying, certain areas of the plates were exposed to the same spectral lines, and to intensities similar to those used for the vaccine virus.

After exposure to the light, small sections of the agar covered with vaccine virus were removed from exposed areas and from unexposed (control) areas, were emulsified with Locke's solution, and both were injected in aliquot doses into the shaved skin of the same susceptible rabbit. The agar plates with the staphylococci were incubated, and the bactericidal effect of the ultra-violet irradiations upon the bacteria was determined by counting the number of colonies in each exposed area and comparing them with the number in unexposed areas of the same size. In this manner figures were obtained which made it possible to express the effect of the light upon the bacteria in percentage of bacteria killed. In dealing with vaccine virus, however, the methods of titration are very crude. Consequently the determinations of the amount of virus inactivated or killed by the light are only relatively accurate, for the readings only indicated a positive or negative skin reaction. Thus the end-point had to be reached each time before a reading could be relied upon, *i.e.*, the virus had to be exposed long enough so that no visible reaction occurred at the site of injection in the skin. Under these conditions, it was impossible to say whether a subinfecting amount of virus remained active after exposure to the light or whether more energy had been used than was necessary to inactivate all of the virus.

A number of experiments were performed in the manner described (1) to determine and (2) to compare the effects that monochromatic ultra-violet light has upon vaccine virus and upon staphylococci. The results are summarized in Table I and graphically presented in Chart 1. In similar bactericidal studies on *S. aureus* carried out within a few months of these experiments closer approximations to the actual amount of incident energy involved in the destruction of all the exposed organisms were obtained. For comparison these figures are included in the table under Staphylococcus II and the curve derived from them has been superimposed upon the cross-hatched areas of the chart.

The upper line in Chart 1 represents the amount of energy in ergs per sq. mm. at each wave-length adequate, or more than adequate, to reduce the quantity of active vaccine virus below the dose necessary to produce a visible lesion in the skin of a susceptible rabbit. The lower line connects observations obtained from experiments in which

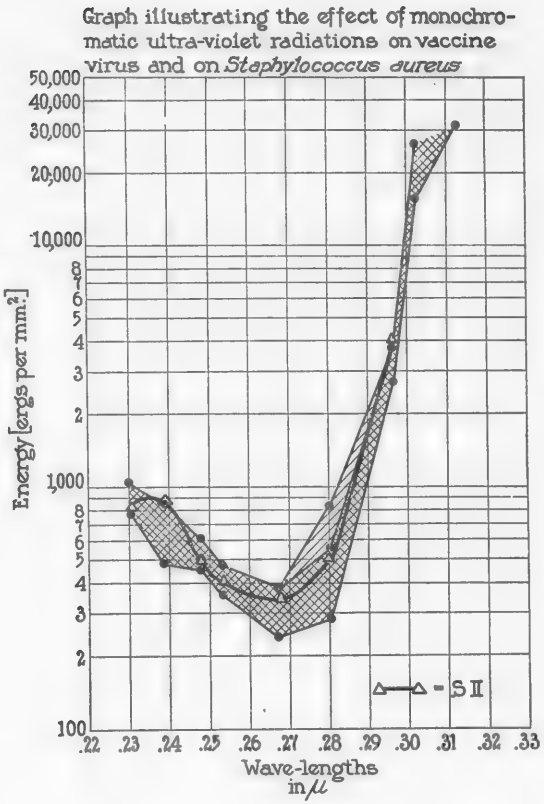


CHART 1. The incident energy required to inactivate all of a given specimen of vaccine virus lies between the upper and lower limits of the area cross-hatched ////. The energy necessary to kill all of the staphylococci falls within the area cross-hatched |||| . It is evident that these areas are identical except in the case of λ 2804. At this wave-length an amount of energy that killed all of the staphylococci failed to inactivate all of the vaccine virus. Only a "lower limit" is given at λ 3126. With the amount of energy used at this wave-length neither vaccine virus nor *Staphylococcus aureus* was seriously injured. It is not known what very large amount of energy, if any, at this wave-length is injurious to the active agents studied. The curve marked S II (*Staphylococcus aureus*) represents a closer approximation of bactericidal energy as found in the experiments of another series.

the amount of energy used was not sufficient to reduce the quantity of active virus to a point below the threshold dose. In regard to staphylococci, it was found (1) that at each wave-length, except one, the amount of energy inadequate to inactivate all the virus killed only from 62 to 76 per cent of the bacteria and (2) that the energy sufficient to inactivate the virus completely also killed all of the staphylococci.

The actual curves of the bactericidal (*Staphylococcus aureus*) and the virucidal (vaccine virus) effects of ultra-violet light lie within the shaded area, which doubtless could be reduced considerably in width by further experiments. Whether the curves for vaccine virus and for

TABLE I.

Amount of Energy at Certain Wave-Lengths Sufficient to Inactivate or Kill Vaccine Virus and Staphylococcus aureus.

Wave-length	Ergs per sq. mm.		
	Vaccine virus	Staphyloccus I	Staphyloccus II
2303–30	1,040	1,040	830
2379–97	856	856	900
2482	619	619	480
2536	480	480	410
2675	356	356	345
2804	858	572	510
2967	3,708	3,708	4000
3022	26,600	26,600	—
3126	30,600 (no effect)	30,600 (no effect)	—

staphylococci are identical cannot be determined with the crude methods available at present for titrating the virus. More significant than the absolute energies involved is the general shape of the curves which might be plotted within the shaded area. A rapid decrease in the required energy between λ 3022 and λ 2804 Ångström units, a nadir at λ 2675, and a rise towards λ 2302, corresponds closely to the curve representing the absorption of ultra-violet light by protein substances, and, in general, to the curves symbolizing various reactions of protoplasm or protein derivatives of protoplasm to light of short wave-lengths. The fact that these curves are similar is interesting and suggestive. Inasmuch, however, as the vaccine virus in a testicular

emulsion is admixed with a great quantity of animal protein to which it is probably adsorbed, one should be careful not to draw final conclusions from such observations as are reported in the present paper.

SUMMARY.

Under the experimental conditions described in the present paper, it was found that the amount of energy required to kill staphylococci at single wave-lengths in the active ultra-violet region was approximately the same as that necessary to inactivate vaccine virus.

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COMPENSATORY HYPERTROPHY OF THE LUNG AFTER UNILATERAL PNEUMECTOMY.

By T. ADDIS, M.D.

(From the Medical Department of Stanford University Medical School, San Francisco.)

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When one kidney or one adrenal cortex is removed the remaining organ becomes larger. This enlargement is due in the main to an increase in the volume of some or all of the cells which compose the parenchyma of the organ, and, since it follows an induced deficiency in total cell mass, the term compensatory hypertrophy has been used to designate the process. But there are some organs which do not manifest any measurable capacity for compensatory hypertrophy. The excision of one testicle is not followed by any significant change in the one which is left (1) and it has been shown that the mass of the medullary portion of the adrenal gland remains the same and the volume of the cells unaltered after unilateral adrenalectomy (2). No satisfactory explanation for this divergence in the reaction of different organs has been advanced and indeed the facts have not yet been defined with sufficient precision to warrant speculation. A quantitative comparison of the varying degrees of compensatory hypertrophy of all the different organs under identical conditions might throw light on the mechanism of the process. But in the meantime even our qualitative information is incomplete for there are no adequate data in regard to the effect of the excision of one lung.[1] The work reported here is an attempt to fill this gap in our knowledge.

[1] Three interesting and decisive studies on the effects of unilateral pneumectomy have appeared from Johns Hopkins University. Heuer and Dunn (3) described the alterations in the position of the thoracic viscera and discussed the surgical aspects of the question. The changes of the alveolar CO_2 and O_2 and in the hemoglobin concentration of the blood were determined by Heuer and Andrus (4). Andrus (5) found that the lung volume had returned to its original value in about 30 days after the operation. There was at first a marked increase in the rate of

When one lung is removed the remaining lung fills the space formerly occupied by the excised lung and pushes the heart close up against the chest wall. There is an obvious enlargement of the organ but it might be wholly due to a greater distension with air, and not at all to any increase in lung tissue. Nor did it seem that the weight of the organ would be a satisfactory measure of the amount of tissue it contained, for all the blood of the body has to pass through the remaining lung, and must add appreciably to its weight. The blood was therefore removed by perfusion with Locke's solution and the quantity of nitrogen in the washed lung was taken as the measure of the mass of lung tissue.

The left lung was removed from female albino rats on the 200th day of age under ether anesthesia. The lung was pulled up by blunt ring forceps into a long retracted intercostal incision while a catgut ligature was passed round the root of the lung with an aneurism needle and tied. The lung was then cut off distal to the ligature. One-third of the animals died from cessation of respiration during the operation. Attempts to remove the right lung were only rarely successful because the right heart dilated when the pulmonary vessels were tied. The surviving rats recovered promptly from the anesthetic and in a very short time were apparently well.

Two groups of controls of the same sex and age were used. In the first no operation was performed, while in the second all the steps of the operation with the exception of the ligature and removal of the lung were completed. The nitrogen content of the right lung of these control rats was compared with the nitrogen content of the remaining right lung of the experimental group.

An interval of from 60 to 61 days was allowed to elapse from the time of operation and then both the experimental and the control animals were anesthetised, several mg. of heparin dissolved in Locke's solution were injected by a needle and syringe into the circulation through the heart, the chest cavity was widely opened, the left auricle incised, a glass cannula thrust into the right ventricle, and Locke's solution run slowly into the still beating heart. As a rule the pink

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flow of blood through the remaining lung. The conflicting histological observations have been summarized by Kawamura (6). He himself observed an increase in the elastic tissue and capillaries in the alveoli of the remaining lung.

lung tissue blanched white at once but in some cases, which were rejected, appreciable quantities of blood were left. When the perfusion fluid ran clear from the left auricle and no more blood could be seen in the lung, the right lung was excised in a uniform manner, cutting the bronchus and vessels as closely as possible to the lung. After blotting on filter paper the entire lung was transferred to a Kjeldahl flask and digested for 3 hours with 30 cc. H_2SO_4, 10 gm. K_2SO_4, and 0.7 gm. Hg.

The left lung of the albino rat was found by Jackson to weigh approximately 0.33 times the total lung weight (7). The results

TABLE I.

Comparison of the Nitrogen of the Right and Left Lungs.

Right lung	Left lung	Both lungs	Ratio: $\frac{\text{Left lung } N_2}{\text{Both lungs } N_2}$
mg.	*mg.*	*mg.*	
19.12	11.94	31.06	0.384
17.56	9.79	27.35	0.358
15.54	7.42	22.96	0.323
15.47	8.49	23.96	0.354
15.28	8.16	23.44	0.348
15.28	8.48	23.76	0.357
14.66	8.07	22.73	0.355
14.62	8.27	22.89	0.361
13.72	7.56	21.28	0.355
Average.....			0.355

given in Table I in which the nitrogen content of the right and left lungs are compared show that the left lung contains on the average 0.36 times the total lung nitrogen.

In the experimental rats, therefore, whose left lung was excised, only about one-third of the total lung tissue was removed, whereas when a kidney, adrenal, testicle, or ovary is excised the total organ tissue is approximately halved. This difference invalidates any comparison of the degree of lung hypertrophy with the degree of hypertrophy in other paired organs. However it will probably be found possible to get about half the lung tissue out by removing first one lobe of the right lung and later the whole of the left lung.

The left lung was removed from twenty-seven rats, but operative deaths, failures to wash blood completely from the lung, and the pneumonia of rats which has been described by Hektoen (8), reduced the number of successful experiments to twelve. The same causes brought the operated control group down to twelve. Pneumonia was found in 24 per cent of the animals. All cases were rejected in which it was present in no matter how slight a degree in either lung.[2]

TABLE II.

Comparison of the Nitrogen Content of the Right Lung in the Control Rats without Lung Excision and in the Experimental Rats Whose Left Lung Had Been Excised.

First controls: no operation	Second controls: left lung exposed	Experimental: left lung excised	Difference between second control and experimental rats
Right lung N_2	Right lung N_2	Right lung N_2	Control = 100 per cent
mg.	*mg.*	*mg.*	*per cent*
19.12	19.40	28.32	+46.0
17.56	17.02	27.74	+63.0
15.54	16.74	26.74	+59.8
15.47	16.47	24.92	+51.5
15.28	16.22	23.11	+42.5
15.28	15.96	22.97	+43.9
14.66	15.62	22.82	+46.2
14.62	15.36	20.72	+35.0
13.72	14.14	20.14	+42.5
	14.02	19.38	+38.2
	13.94	18.33	+31.5
	13.67	18.33	+34.1
Average. 15.7	15.7	22.8	+45

The results are given in Table II, in which the absolute amounts of N_2 found are recorded and in Table III, where the N_2 is expressed

[2] In one control animal it happened that the whole of the left lung was consolidated and reduced to about one-fourth of its usual size, while the right lung was free from pneumonia. A nitrogen determination showed that the right lung contained 33.78 mg. N_2, more than twice the average nitrogen content of the right lung of the controls. In several other instances of unilateral disease a high N_2 content was found in the other lung so that it would seem that an increase in the mass of the remaining healthy lung tissue occurs as a result of lung disease in the rat.

as mg. per sq. dm. of body surface (body surface = 11.4 times the two-thirds power of the body weight).

In both control groups the amount of N_2 in the right lung averaged 15.7 mg., while the average N_2 of the one lung rats was 22.8 mg. Relatively to body surface the controls had 3.5 and 3.8 mg. N_2 per sq. dm., while the experimental rats had 5.5 mg. N_2 per sq. dm. of

TABLE III.

Comparison of the Nitrogen Content of the Right Lung Per Sq. Dc. of Body Surface in the Control Rats without Lung Excision and in the Experimental Rats Whose Left Lung Had Been Excised.

First controls: no operation	Second controls: left lung exposed	Experimental: left lung excised	Difference between second control and experimentol rats
Right lung N_2	Right lung N_2	Right lung N_2	Control = 100 per cent
mg. per sq.dm.	*mg. per sq.dm.*	*mg. per sq.dm.*	*per cent*
4.64	4.91	6.72	+36.9
3.96	4.05	6.31	+55.8
3.75	4.05	6.28	+55.1
3.65	4.00	6.27	+56.8
3.38	3.86	6.15	+59.4
3.23	3.81	5.82	+52.8
3.15	3.78	5.18	+37.0
3.08	3.64	5.04	+38.5
2.98	3.61	4.78	+32.5
	3.58	4.58	+28.0
	3.43	4.55	+32.6
	3.41	4.36	+27.9
Average..3.5	**3.8**	**5.5**	**+43**

body surface. When the operated control and experimental results are arranged in order of magnitude and compared individually there is in every instance a marked increase in the N_2 of the one lung rats. The average increase is 45 per cent when the N_2 is given in absolute terms and 43 per cent when it is expressed relatively to body surface.

CONCLUSION.

In the albino rat the removal of the left lung, which constitutes about one-third of the total lung tissue, is followed by an average

increase of more than 40 per cent in the mass of the remaining lung, as estimated from the nitrogen content of the almost blood-free tissue.

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THE PRECIPITIN REACTION OF ANTIPNEUMOCOCCUS SERA.*

I. The Precipitin Index.

By HARRY SOBOTKA, Ph.D., and MAE FRIEDLANDER.

(From the Department of Bacteriology, New York University and Bellevue Medical College, New York.)

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The study of the precipitating reaction between antipneumococcus sera and the soluble specific substance derived from the three fixed types of *Diplococcus pneumoniæ* promises a quantitative method for measuring the antibodies present in antipneumococcus sera. The relative simplicity in the chemical composition of one of the constituents, namely the soluble specific carbohydrate, paves the way for making this immunological phenomenon better understood.

There is little doubt that the mutual compensation of electrical charges between two reacting colloids causes the phenomenon of precipitation (1, 2). So far colloidal chemistry offers an explanation for the mechanism of the reaction but not for the cause of its specificity toward that part of the colloidal serum constituents commonly known as antibodies. Avery, Heidelberger, and Goebel (3) demonstrated that the type specificity of the antigen is accounted for by distinct differences in chemical composition between the soluble specific substance of the three fixed types. The carbohydrates with their variability of configuration are apt to cause great physiological specificity, *e.g.* in the enzymatic hydrolysis of saccharides.

On the other hand, the knowledge of the chemical nature or even of the concentration of the active specific principle of the other participant, the antibody, is so obscure that a differentiation of types from this angle seems futile at the present time.

We endeavored to establish mathematical laws for the precipitin

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reaction in the various types of pneumonia and thus from the dynamics of the specific reaction itself to obtain information concerning specificity.

When one adds various dilutions of antigen (precipitinogen)[1] to a constant dilution of antibody (precipitin) precipitation occurs until a certain dilution of antigen is reached. In the same way, when varying dilutions of antibody (precipitin) are added to a constant dilution of antigen (precipitinogen) precipitation occurs until a certain dilution of antibody is reached. The results of these reactions may be tabulated in charts as in Table I.

TABLE I.

Precipitin Reaction of Antipneumococcus Serum 32, Type I.

Dilution of serum	Dilution of soluble specific substance in millions											
	0.8	1.6	3.2	6.4	12.8	19.2	25.6	38.4	51.2	76.8	102.4	153.6
10	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	−
30	+	+	+	+	+	+	+	+	+	+	−	−
40	+	+	+	+	+	+	+	+	+	−	−	−
60	+	+	+	+	+	+	+	−				
80	+	+	+	+	+	+	±	−				
120	+	+	+	+	+	−	−	−				
160	+	+	+	+	+	−	−	−				
200	+	+	+	+	−	−						
240	+	+	+	+	−	−						
320	+	+	+	±	−	−						
400	+	+	+	−	−	−						
480	−	−	−	−	−	−						

The sensitivity of this precipitation, as determined by the last plus sign (positive precipitation) in any horizontal or vertical row, may be computed by multiplication of the concentration of the soluble specific substance by the concentration of the antibody. Where the antibody concentration is increased the antigen may be proportionally decreased and still give a positive reaction; an eightfold dilution of an antibody solution, for example, requires an eight times greater concentration of the antigen to give precipitation compared with the antigen

[1] The terms "antigen" and "precipitinogen" are used in the following in the sense of a chemical reagent; no implication is made as to immunizing action (4).

concentration necessary to precipitate the original antibody solution. The mathematical expression for this inverse proportionality is the constancy of the above product which can be verified for the wide range of serum dilutions from 1:5 to 1:500. The product is constant within this range just like the so called "solubility product" in the case of inorganic ions forming insoluble compounds. An "ionic product" can be calculated for a mixture of solutions containing barium ions and sulfate ions by multiplying the concentration of the two. When this surpasses a certain value, commonly termed the solubility product, more barium sulfate is formed than can be kept in a dissolved state and precipitation occurs. The mathematical law for the appearance of the insoluble precipitate as observed in this example may be compared, to a certain extent, to that applying to the appearance of insoluble precipitate when antiserum is added to antigen. The mechanism of this combination is different from the barium sulfate formation in so far as the composition of the precipitated compounds is not constant as to the amounts of the two constituents. Colloidal reactions are not stoichiometrical and precipitates vary as to their composition as indicated by their different gross appearance. When the serum is in excess a voluminous precipitate is formed consisting of many very fine particles which stay in suspension for some time after shaking. On the other hand an excess of soluble specific carbohydrate will cause a precipitation of larger individual flakes which settle to the bottom more readily and stick together.

By testing the supernatants it may be shown that an excess of soluble specific substance removes the antibody beyond the point where it can be detected, but an excess of antibody fails to remove the soluble substance to this extent (see Table VIII, page 69) (12,13,14).

Absolute values for the solubility products of the specific precipitates cannot be given because the absolute value for one constituent only can be inserted. The soluble specific substance has a constant chemical composition which in pure state exhibits constant immunological activity. It may be used as a reproducible standard in evaluating the relative concentration of the antibody which is measured by the sensitivity of the precipitation with the former at maximum dilutions. The absolute concentration and activity of the antibody, however, are still unknown because it has not as yet been demonstrated free from other components of the serum.

It seems practical to substitute the exceedingly small values of the solubility product by their reciprocals. These are computed by multiplying the dilutions instead of the concentrations. As these figures were higher than a million their one-millionth part was used as the *Precipitin Index*. If this index were constant throughout each test it would be sufficient to look for it in one dilution of one constituent and to vary only the dilution of the other one. Different factors, however, bring about irregularities in the precipitin reaction.

As precipitation according to the accepted assumption is due to the mutual compensation of the electrical charges of a positive and a negative colloid, a large excess of either one is able to keep the colloid of opposite charge in solution. On this account a considerable amount of either antigen or antibody will fail to precipitate in the presence of an excessive concentration of the other. A phenomenon of a similar type, usually termed pro-zone, is met with in the agglutination of *B. typhosus* (5) and of Pneumococcus; a high concentration of serum fails to cause agglutination with a concentration of antigen which gives a positive reaction with lower serum concentrations. The opposite phenomenon was described for precipitation in Type II pneumonia by Morgan (6), *viz.* a rather high concentration of soluble specific substance did not react with antiserum in its lower concentrations although the latter did react with smaller amounts of the soluble specific substance. We designate this reversed action the post-zone. Zones are more frequently observed in Type III than in Type I.

Consideration of the ratio between soluble specific substance and serum reveals that the pro-zone occurs in the test-tubes where this proportion ranges between 80,000 and 320,000 for Type III and between 300,000 and 1,600,000 for Type I. A similar figure can be derived for the post-zone ranging between 1,250 and 6,250 for Type III and between 400 and 2,400 for Type I. Although both the pro-zone and post-zone do not appear in all sera, we are able, however, to observe both zones in the same serum. In such cases the diagram suggests a certain zone of positive precipitation between the limits given in the above figures.

The quotients sss dilution / serum dilution or serum concentration/ sss concentration for the post-zone in Types I and III are about one-tenth of the corresponding values for the point at which the antibody

is entirely removed by precipitation (*cf.* Table VIII). A ten times greater amount of antigen than is necessary to precipitate one unit of antibody is required to keep it in solution.

The absolute concentration of antibody in serum is unknown. Tentatively let us assume that the concentration of the precipitin anti-

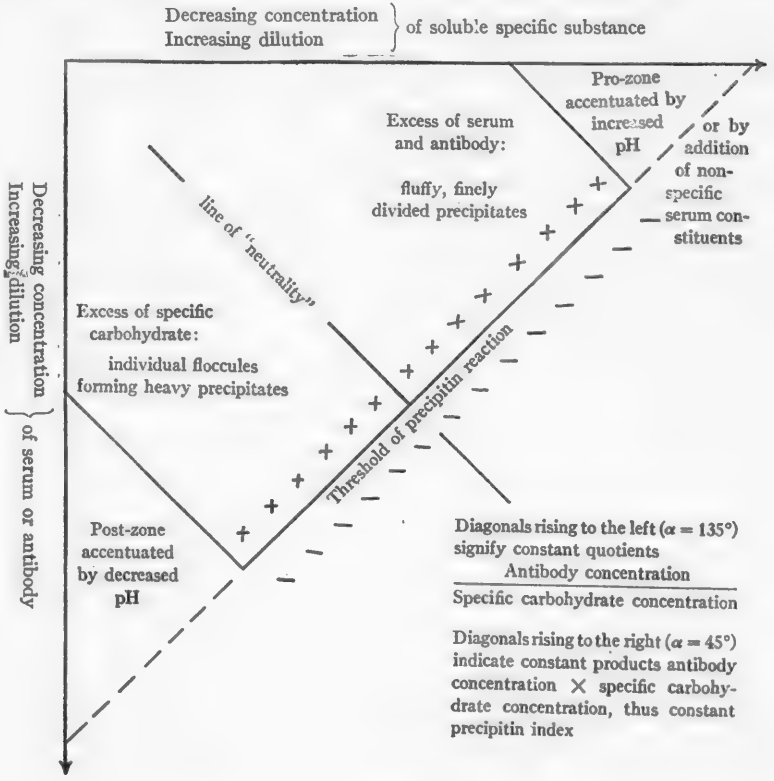
Decreasing concentration
Increasing dilution
of soluble specific substance
Pro-zone accentuated by increased pH
or by addition of non-specific serum constituents
Excess of serum and antibody:
fluffy, finely divided precipitates
line of "neutrality"
Excess of specific carbohydrate:
individual floccules forming heavy precipitates
Threshold of precipitin reaction
Decreasing concentration
Increasing dilution
of serum or antibody
Post-zone accentuated by decreased pH
Diagonals rising to the left (α = 135°) signify constant quotients
Antibody concentration / Specific carbohydrate concentration
Diagonals rising to the right (α = 45°) indicate constant products antibody concentration × specific carbohydrate concentration, thus constant precipitin index

FIG. 1.

body in the serum is 1 in 20,000;[2] then the quotients antibody/sss attain 1/20,000 of the values antiserum/sss given above as character-

[2] According to a private communication from Dr. F. M. Huntoon a value between 0.054 and 0.018 per cent may be assumed for the percentage of antibody in the usual antipneumococcus sera.

istic of the two limiting zones. Hence for Type I the compound is insoluble between the limits of 15 to 80 parts antibody to 1 part SSS on the one side, and of 1 part antibody to 8 to 50 parts SSS on the other. For Type III the compound is insoluble in a fourfold to sixteenfold excess of either antibody or specific carbohydrate.

The range of insolubility in Type III seems from the above figures to be limited to a much narrower variation in the proportion of precipitin to precipitinogen than in Type I. In Morgan's experiment

TABLE II.

Ratio $\frac{\textit{Concentration of Antiserum}}{\textit{Concentration of SSS}}$ *for Pro- and Post-Zones.*

Zone and type	Range of variation	Extreme values		Number of observations	Average quotient
		Lowest	Highest		
Pro I	320,000–1,600,000	**200,000** 215,000	3,200,000	13	900,000
Post I	400–2,350	400	**5,000**	11	1,500
Pro III	80,000–320,000	**50,000** 60,000	2,560,000 1,280,000 960,000 640,000 (twice) 480,000	26 (18)	350,000 (210,000)
Post III	1,250–6,250	1,000	**40,000** 20,000 10,000	21 (17)	6,000 (3,000)

with Type II the figures indicate that this variation lies between I and III. These differences are partially if not entirely due to the differences in acid equivalents of the soluble specific substances. The quotient antibody/SSS for the post-zone in Type III is three times that in Type I. In other words it requires one-third the excess of soluble specific substance III than I to prevent the homologous precipitation. According to their acid equivalents—340 for Type III and at least 600 for Type I—these solutions are about equimolecular.[3] Similar

[3] A report of experiments on the equivalent weight of soluble specific substance, Type I, will be given in another place.

considerations cannot yet be applied to the antibody because of the presence of a non-specific factor in the serum. Whether this factor whose manifestations will be discussed in the following paragraphs is an independent fraction of the serum globulin or associated with the antibody proper is a problem for future investigation.

Addition of Non-Specific Serum Fraction (*cf.* 7).—The antigenic soluble substance constitutes only a small fraction of the precipitate which by itself is only a fraction of the total solids present in the reacting fluids. By far the greater part of the precipitate originates in the serum. It is difficult to imagine that this overwhelming portion should be endowed with as high a degree of specificity per unit of weight as is the specific carbohydrate, unless a single specific group of the antibody is associated with a large protein molecule; in other words the antibody equivalent is very high. The following facts suggest that the association between the antibody proper and its protein carrier can be broken without irreversible inactivation of the antibody group: refined fractions obtained from antisera by the ammonium sulfate methods and containing the full amount of protective antibody, as proven by injection into the blood stream, at times do not show the precipitin reaction. This ability could be restored in some of our experiments by addition of normal serum.

A similar though less striking effect was observed with other purified antibody solutions and several original antisera, where, by the addition of normal serum as well as of the globulin fractions of normal serum or heterologous antisera, an inconspicuous precipitin reaction was increased as to its amount and as to its extent in the series of dilutions. Another general observation points to the complex nature of the antibody: the disappearance of the precipitin reaction in serum concentrations of 1 to 500 and below compared with the high sensitivity for low antigen concentration may be explained by the multimolecular character of the reaction on the part of the serum.

In Serum 32, Type I, the addition of normal horse serum increased the sensitivity when the concentration of antibody was low, but it reduced the sensitivity causing a pro-zone when the concentration of the antiserum was high (Table III).

A general increase of the precipitin indices upon addition of normal serum to Serum 68, Type I, is recorded in Table IV.

TABLE III.

Precipitation of Antipneumococcus Serum from Horse 32, Type I.

A. With Addition of 10 Per cent Normal Horse Serum.

Dilution of antiserum	Dilutions of soluble specific substance in millions											
	0.04	0.2	0.4	0.8	1.6	3.2	6.4	12.8	25.6	38.4	51.2	102.4
20							+	+	+	±	−	−
40							+	+	+	±	−	−
60							+	+	+	+	−	−
80							+	+	+	±	−	−
100						+	+	+	+	−	−	
120						+	+	+	±	−	−	
160					+	+	+	+	−	−		
200					+	+	+	+	−	−		
240					+	+	+	±	−	−		
280					+	+	+	±	−	−		
320				+	+	+	+	−	−			
400				+	+	+	+	±	−			
480			+	+	+	+	+	−				
600			+	+	+	+	±	−				
800		+	+	−	−	+	+					
1200		+	−	−	+	+						
2400	−	−	−	−								

B. Without Addition of Normal Serum.

20							+	+	+	+	+	+
40							+	+	+	+	±	−
60							+	+	+	+	−	−
80							+	+	+	−	−	−
100						+	+	+	±	−	−	
120						+	+	+	±	−	−	
160					+	+	+	+	−	−	−	
200					+	+	+	+	−	−	−	
240					+	+	+	+	−	−	−	
280					+	+	+	−	−	−		
320					−	+	+	−	−	−		
400			−	−	−	−	−	−	−			
480			−	−	−	−	−	−	−			
600			−	−	−	−	−	−				
800			−	−	−	−	−	−				
1200		−	−	−	−	−						
2400		−	−	−	−	−						

An experiment tending to confirm the significance of this factor is the dominance of the pro-zone when two sera, only one of which shows a pro-zone, were used in a mixture of equal amounts. The result as given in Table V was not an average as one might expect, but practically the same as in the serum with the pro-zone. The significance of this frequently occurring condition upon the testing of pooled serum and similar mixtures is obvious.

The influence of buffers on the sensitivity and on the zonal phenomena of the precipitin test was investigated (*cf.* 6, 8). Of course, serum itself is a buffer. The pH of a serum buffer mixture will as a rule be found between the pH of the buffer and the pH of the serum. The value will approach the more concentrated and better buffer of the two.

TABLE IV.

Precipitin Indices for Serum 68, Type I, with and without Addition of 10 Per Cent Normal Horse Serum.

Dilution of antiserum	Precipitin index	
	With 10 per cent normal serum	Without 10 per cent normal serum
10	60	<20
20	80	<40
40	320	80
80	320	160
160	480	640
320	960	480

In a special experiment the influence of the specific precipitation on the pH of the serum was determined. As expected from the minute amount of precipitate (see page 63), it was found either constant or showed slight and irregular changes only.[4]

The following conclusions were drawn. Phosphates outside the natural range of reaction impair the sensitivity; citrate in the range used in our experiments does not act in this way. The same type of buffer at different pH causes the pro-zone to disappear and the post-zone to appear when going to the acid side. The development of a post-zone renders the reaction less sensitive. Although the actual

[4] E. F. Hirsch (9) observed slight changes to the alkaline side in a rabbit anti-sheep precipitin system.

changes in pH are slight, they increased the power of the soluble specific substance to keep the antibody in solution, thus causing a post-zone. This can be attributed to the fact that the dissociation of the acid soluble carbohydrate is increased toward low pH making it possible to reach more readily an excess of the specifically active anions. When the reaction approaches the alkaline side, formation of electropositive

TABLE V.

Precipitin Test of Two Type III Sera, 125 and 126 and of Their Mixture.

No. and dilution of serum	Dilution of soluble specific substance in millions										P.I.
	0.4	0.8	1.2	1.6	3.2	4.8	6.4	9.6	12.8	25.6	
No. 125											
5	+	+	+	+	+	−	−	−	−	−	(16)
10	+	+	+	+	+	−	−	−	−	−	(32)
20	+	+	+	+	+	+	+	±	−	−	160
40	+	+	+	+	+	±	−	−	−	−	160
60	+	−	−	−	−	−	−	−	−	−	(24)
No. 126											
5	+	+	+	+	+	+	+	+	+	−	(64)
10	+	+	+	+	+	+	+	+	−	−	(96)
20	+	+	+	+	+	+	+	+	−	−	192
40	+	+	+	+	+	+	±	−	−	−	224
60	+	+	+	+	+	±	−	−	−	−	240
No. 125+126											
5	+	+	+	+	+	−	−	−	−	−	(16)
10	+	+	+	+	+	−	−	−	−	−	(32)
20	+	+	+	+	+	+	±	−	−	−	112
40	+	+	+	+	+	+	±	−	−	−	224
60	+	−	−	−	−	−	−	−	−	−	(24)

For parenthesized figures see Table VI of following paper.

ions from the antibody which probably has an ampholytic nature (10) is increased thus accounting for the more prompt appearance of a prozone by overcompensation of the negative charges of the anions of the soluble specific substance.

Practical applications of these facts are treated in the succeeding paper.

EXPERIMENTAL PART.

The antigenic material employed in our tests consisted of the type specific soluble substances prepared from broth cultures of pneumococci and purified by

TABLE VI.

Precipitin Index of Antipneumococcus Serum 32, Type I, from Test Recorded in Table I.

Dilution of serum	Greatest dilution of sss giving a+ (or ±) precipitation	Precipitin index by multiplication of corresponding serum and sss dilutions	
	millions		
10	102.4	1024	
20	102.4	2048	
30	76.8	2304	
40	51.2	2048	
60	25.6	1536	
80	(25.6)	1792	1722
120	12.8	1536	
160	12.8	2048	
200	6.4	1280	
240	6.4	1536	
320	(6.4)	1536	
400	3.2	1280	
480	<0.4	<192	
Dilution of sss	**Greatest dilution of serum giving a + (or ±) precipitation**		
millions			
0.8	400	320	
1.6	400	640	
3.2	400	1280	
6.4	(320)	1792	
12.8	160	2048	
19.2	80	1536	
25.6	(80)	1792	1820
38.4	40	1536	
51.2	40	2048	
76.8	30	2304	
102.4	20	2048	
153.6	<10	<1536	

repeated precipitations; their ash was less than 0.5 per cent, the nitrogen of the Type I substance was 4.5 per cent by Kjeldahl.

Dilutions were made ranging from 1:250,000 to 1:150,000,000 in the case of

Type I and from 1:100,000 to 1:50,000,000 with Type III. The increase in the magnitude of these solutions was in geometric progression with an increment of 2, or 3/2 and 4/3 alternatingly.

The sera used were thoroughly centrifugalized at 2,000 to 2,400 revolutions per minute for 1 hour and the supernatant filtered before making up the dilutions. It was necessary to take these precautions because in many of the sera a sediment occurs which might easily be mistaken for a positive "thread" reaction. Normal serum, too, before centrifugalization gave what seemed to be positive results. Controls consisted of various dilutions of serum in physiological salt solution. Wherever these were not negative the test was discarded.

TABLE VII.

Determination of Precipitin Index of Serum 90, Type I.

Dilution of serum	Dilutions of sss Type I in millions											Precipitin index
	1	2	4	8	16	24	32	48	64	96	128	
5	+	+	+A	+	+	+	+	+	+	+	+	640
10	+	+	+	+	+	+	+	+	−	−	−	480
20	+B	+	+	+	+	+	+	+	−	−	−	960
40	+	+	+	+	+	−	−	−	−	−	−	640
60	+	+	+	+	+	−	−	−	−	−	−	960
80	+	+	+	+	−	−	−	−	−	−	−	640
160	+	+	+	−	−	−	−	−	−	−	−	640
240	+	+	±	−	−	−						720
320	+	+	−	−	−	−						640
480	−	−	−	−	−	−						(<480)
Average												725

For A and B see Table XII.

Series of twelve tubes (2½ by ⅝ inches) were arranged in racks and marked with the figures of the increasing dilutions of the soluble specific substance of which 0.5 cc. was used. To each was added 0.5 cc. of the dilution of the serum. Thus each rack contained tubes with varying concentrations of soluble specific substance and a constant concentration of serum. These, together with controls, were placed in a water bath with 37°C. for 2 hours and then put into the ice box until the following morning when the reactions were read and recorded.

If a flaky precipitate was not visible at first the tubes were spun for a thread reaction. The use of ++, +++, etc. was discarded because it is impossible to indicate from the appearance of the precipitate the quantity actually present (6, 11). Doubtful tubes were recorded by the sign ±.

The method of calculating is illustrated in Table VI. The last dilution of the soluble specific substance giving a + reaction was multiplied by the serum dilution

used in this particular test (rack). If the last reaction is doubtful—such ± reactions are recorded in the tables by parenthesized figures—the average value between it and the decidedly positive reaction to its left was inserted. Similar figures were obtained when the limit of sensitivity was taken from the vertical rows of equal sss concentration instead of the horizontal. An average was taken over the range of high sensitivity thus eliminating zonal effects.

In Table I and in Table VI antiserum from Horse 32, Type I, was used. The precipitin index — P.I. — from the horizontal rows was

TABLE VIII.

Precipitin and Precipitinogen in Supernatants of Precipitates from Polyvalent Antipneumococcus Serum 3335 with Soluble Specific Carbohydrates of Types I and III.

Precipitate formed from dilution of		To corresponding supernatant added equal amount of												
		Dilution of sss (millions)										Dilution of serum		
Serum	sss I			0.125	0.25	0.5	1	2	4	8	16	10	20	40
2.5	4,000,000			+	+	+	+	+	+	±	−	±	−	−
5	2,000,000			+	+	+	+	+	±	−	−	±	±	−
10	1,000,000			+	+	+	+	±	−	−	−	+	±	±
20	500,000			+	+	+	±	−	−	−	−	+	+	−
40	250,000			−	−	−	−	−	−	−	−	+	+	+
	sss III	0.025	0.05	0.1	0.2	0.4	0.8	1.6	3.2	6.4	12.8			
2.5	800,000	+	+	+	+	+	+	+	+	+	±	±	±	−
5	400,000	+	+	+	+	+	+	+	+	+	−	±	±	±
10	200,000	−	−	−	−	−	−	−	−	−	−	+	+	±
20	100,000	−	−	−	−	−	−	−	−	−	−	+	+	+
40	50,000	−	−	−	−	−	−	−	−	−	−	+	+	+

1,720, from the vertical rows 1,820. As a rule the P.I. was computed from the horizontal rows.

Table VII is another very regular test in which Antiserum 90, Type I, was used with a resulting P.I. of 725.

Serum 3335, a polyvalent serum obtained from a horse immunized against all three types of pneumonia gave regular results and was used to obtain approximate information as to the equivalence of the two reagents.

10 cc. of serum in concentrations of $2\frac{1}{2}$, 5, 10, 20, and 40 per cent were

mixed with 10 cc. of soluble specific substance Type I in dilutions of 4, 2, 1, $\frac{1}{2}$, $\frac{1}{4}$ million respectively. Another series with the same serum dilutions were made with soluble specific carbohydrate Type III in dilutions of 800,000, 400,000, 200,000, 100,000, and 50,000. The precipitates were centrifugalized after the usual incubation and the supernatants tested for remaining antibody or antigen. In the first four supernatants of the experiments with Type I where the serum

TABLE IX.

Examples for Pro-Zone and Post-Zone of Precipitation with Antisera from Type III Horses.

No. and dilution of serum	Dilution of soluble specific substance									Quotient $\frac{\text{Serum}}{\text{sss}}$
	Millions									
	0.2	0.4	0.8	1.2	1.6	3.2	4.8	6.4	9.6	For pro-zone
(*a*) No.302										
20	+	+	+	+	+	−	−	−	−	80,000
40	+	+	+	+	+	+	−	−	−	80,000
80	+	+	+	+	+	+	+	+	−	80,000
160	+	+	+	+	+	+	±	−	−	−
320	−	−	−	−	−	−	−	−	−	−
	Thousands									For post-zone
	24	32	40	48	64	96	128	256	512	
(*b*) No.127										
20	+	+	+	+	+	+	+	+	+	−
40	+	+	+	+	+	+	+	+	+	−
60	−	−	±	±	+	+	+	+	+	667
80	−	−	−	−	−	+	+	+	+	800
120	−	−	−	−	−	−	+	+	−	800
160	−	−	−	−	−	−	−	−	−	−

concentration was 25,000 or more times that of the soluble specific substance and in the first two supernatants of the Type III series where this excess was 80,000 fold, antibody was present. The subsequent combinations with a serum/sss quotient of 6,250 for Type I and 20,000 for Type III did not contain detectible amounts of antibody.[5]

An analogous experiment was performed, testing the supernatant

[5] Morgan (6) gives a ratio 1:10,000 to 1:20,000 soluble specific substance: antiserum for maximum precipitation in the Type II system.

with the original serum. Only those supernatants found free from antibody gave a considerable reaction with soluble specific substance; the others revealed the presence of slight amounts of non-precipitated antigenic substance by a faint reaction (12, 13, 14).

The ten original precipitates were resuspended in physiological salt solution and combined with undiluted serum or 0.1 per cent soluble specific substance. Some of the precipitates changed their appearance but none redissolved.

Table IX (*a*) gives a pro-zone for pooled Serum 302, Type III, the zone being characterized by a serum/sss quotient of 80,000. An ex-

TABLE X.

Precipitin Test of Antibody Preparation 102, Type I, with Soluble Specific Substance in Dilutions 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 38.4, 51.2, 102.4 Million, in Presence of 10 Per Cent Normal Horse Serum.

Dilution of preparation	Greatest dilution of sss giving + (or ±) reaction	Precipitin index		Quotient $\frac{\text{Serum}}{\text{sss}}$
	millions			*pro-zone*
80	(51.2)	3584		560,000
120	25.6	3072		215,000
160	51.2	8192		320,000
200	51.2	10,240		
240	(51.2)	9216		
320	25.6	8192	7170	
400	(12.8)	3840		
480	12.8	6144		
600	6.4	3840		
800	(12.8)	7680		
1200	(1.6)	1440		

periment for the post-zone phenomenon is given in Table IX (*b*) carried out with Serum 127, Type III, against the homologous specific substance. The simultaneous occurrence of a post-zone (serum/sss = 3,750) and a pro-zone (serum/sss = 960,000) are shown in Table XIV (*a*) (page 75).

Preparation 102 from pooled antisera Type I precipitated in the presence of 10 per cent normal horse serum as shown in Table X. Without this addition it did not exhibit any precipitin power at all.

The precipitation as a whole was not very strong but reached high dilution. There is no doubt as to the existence of a pro-zone. Table III, for which the numerical values of the P.I. are given in Table XI presents the influence of normal serum on Antiserum 32; its sensitivity is increased and a pro-zone with a serum/sss of 600,000 and 800,000

TABLE XI.

Precipitin Test of Antiserum 32, Type I, Same Sample as Recorded in Table III.
(A) With Addition of 10 Per Cent Normal Horse Serum.
(B) Without This Addition.

Dilution of antiserum	Greatest dilution of sss giving +		Precipitin index		Quotient $\frac{\text{Serum}}{\text{sss}}$	
	(A)	(B)	(A)	(B)	(A)	(B)
20	(38.4)	102.4	640	2048	1,600,000	
40	(38.4)	(51.2)	1280	1792	800,000	
60	38.4	38.4	2304	2304	640,000	
80	(38.4)	25.6	2560	2048		
100	25.6	(25.6)	2560	1920		
120	(25.6)	(25.6)	2304	2304		
160	12.8	12.8	2048	2048		
200	12.8	12.8	2560	2560		
240	(12.8)	12.8	2304	3072		
280	(12.8)	6.4	2688	1792		
320	6.4	6.4 **1.6**	2048	2048		5000
400	(12.8)	<0.4	3840	<160		
480	6.4		3072			
600	(6.4)		2880			
800	6.4 **1.6**		5120		2,000	
1200	3.2 **0.8**		3840		667	
2400	<0.04		<96			

The underscored bold faced figures give greatest dilution of sss giving − reaction in post-zone.

is produced. Another example is given in Table IV, page 65, showing the influence of 10 per cent normal horse serum on Type I Antiserum 68.

The influence of buffers on the actual pH of Type I Antiserum 90 was measured potentiometrically and it was found that the deviations from the pH of the buffer itself are smaller in the range where the buffer has the highest buffer value (15). For the same buffer mixture a

smaller deviation was caused by the addition of 5 per cent serum than of 40 per cent serum.

Precipitation of these mixtures with soluble specific substance using

TABLE XII.

pH of Mixtures of Equal Amounts of Buffers and Serum 90, Type I, and Influence of Precipitation upon pH of Buffered Serum for Combinations Marked A and B in Table VII.

Buffer		pH by potentiometric determination				
		Buffer alone	Serum dilution 2.5	A Serum dilution 5 sss dilution 4 million	Serum dilution 10	B Serum dilution 20 sss dilution 1 million
Phosphates (M/15)						
Primary	Secondary					
10.0	0.0	4.56	6.23	6.19	5.68	—
9.5	0.5	5.53	6.37	6.37	—	—
9.0	1.0	5.83	6.27	—	6.02	5.92
7.0	3.0	6.38	6.71	6.57	6.43	6.39
4.0	6.0	6.91	7.08	7.17	6.89	6.90
1.5	8.5	7.46	7.55	7.61	7.42	7.24
0.5	9.5	7.88	7.88	7.60	7.60	7.33
Citrate NaOH (N/10)						
10.0	0.0	4.92	5.20	5.30	4.98	4.97
8.0	2.0	5.35	5.72	5.77	5.37	5.42
6.0	4.0	5.98	6.72	7.05	6.24	6.24
Borate (N/10)		9.10	—	—	9.00	—
Serum 90		Undiluted 7.72	—	—	7.66 / 7.58	—

Precipitates in phosphate mixtures more voluminous, with citrate "10.0" very slight.

a dilution of 4 million for serum dilution 2.5 and 1 million for serum dilution 20 caused slight variations as given in Table XII.

If a certain phosphate mixture is added to varying concentrations of serum the pH for low serum concentration will approach the original

TABLE XIII.

Influence of Phosphate Buffers upon Precipitin Test of Antipneumococcus Serum 3534, Type III. (*a*)

Greatest dilutions of soluble specific substance III giving + (or ±) precipitation, in millions. Dilutions used: 0.3, 0.4, 0.6, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2.

A. Final concentration of phosphate N/20 throughout the test

Dilution of serum	pH of buffer	4.6	5.5	6.1	6.8	7.3	8.0
	pH of mixture with serum dilution 10	–	–	–	7.13	7.38	7.45
10		(25.6)	12.8	25.6	(6.4)	3.2	3.2
20		6.4	(25.6)	(6.4)	6.4	3.2	3.2
40		3.2	6.4	3.2	(3.2)	<0.8	0.8
80		(3.2)	1.6	0.8	(3.2)	<0.2	<0.2
160		<0.2	<0.2	<0.2	<0.2	–	–
80 post-zone	Greatest dilution of SSS giving – (or ∓) precipitation	0.8	0.2	0.4	(3.2)	–	–
40		–	–	–	(0.8)	–	–
Serum/SSS for post-zone		10,000	2,500	5,000	20–40,000	–	–

B. Original serum diluted with N/4 phosphate

Dilution of serum	Concentration of buffer	pH of buffer	(*b*) 4.6	5.5	6.1	6.8	(*c*) 7.3
		pH of mixture with serum dilution 10	6.37	6.50	6.63	7.05	7.53
5	N/20		25.6	12.8	51.2	12.8	(12.8)
10	N/40		(25.6)	12.8	51.2	12.8	12.8
20	N/80		6.4	6.4	(12.8)	6.4	6.4
40	N/160		6.4	6.4	6.4	(6.4)	6.4
80	N/320		1.6	(3.2)	(3.2)	(3.2)	3.2
160	N/640		(1.6)	1.6	(1.6)	1.6	3.2
160 post-zone		As above	0.2	–	0.4	(0.2)	–
Serum/SSS for post-zone			1,250	–	2,500	1,000	1,920,000 for pro-zone

(*a*) For test of Serum 3534 without buffer see Table XIV (*a*).

(*b*), (*c*) Compare Table XIV (*b*, *c*).

pH value of the phosphate. If this pH is extreme no reaction takes place. This occurred with phosphate buffers of a pH less than 5.0 and more than 6.5 for serum concentrations of 1:40 and below. Even in higher serum concentrations alkaline phosphates will impair the sensitivity, as seen in Table XIII, *A*, which gives the effect of phosphates upon precipitation of Serum 3534, Type III.

TABLE XIV.

Influence of Hydrogen Ion Concentration on Precipitin Reaction of Type III Serum 3534.

Dilution of serum	Dilution of soluble specific substance in millions									Precipitin index
	0.2	0.4	0.8	1.6	3.2	6.4	12.8	25-6	51.2	
(*a*) Without buffer										
5				+	+	±	−	−	−	24
10				+	+	+	±	−	−	96
20			+	+	+	+	−	−		128
40			+	+	+	±	−	−		192
80	−	±	+	+	−	−				128
160	−	−	−	−	−	−				−
(*b*) Phosphate 4.6										
5				+	+	+	+	+	−	128
10				+	+	+	+	±	−	192
20			+	+	+	+	−	−		128
40			+	+	+	+	−	−		256
80	+	+	+	+	−	−				128
160	−	+	+	±	−	−				192
(*c*) Phosphate 7.3										
5				+	+	+	±	−	−	48
10				+	+	+	+	−	−	128
20			+	+	+	+	−	−		128
40			+	+	+	+	−	−		256
80	+	+	+	+	+	−				256
160	+	+	+	+	+	−				512

The pH can, however, be maintained constant in a test by diluting the buffer parallel to the serum. Thus, in Table XIII, *B*, where the original serum is diluted *after* addition of $\frac{1}{4}$ N phosphates even the slight variations in the resulting acidity show pronounced effects on the diagram. The sensitivity of the reaction was increased toward the

alkaline side and at the same time a post-zone characteristic of the serum disappeared. On the acid side a higher sensitivity in the greater serum concentrations suppressed the pro-zone.

When using citrate of pH 5.0 a post-zone appeared and the sensitivity increased for a Type III serum as compared with a control without any buffer or with serum phosphate mixture more alkaline than the citrate.

TABLE XV.

Influence of Buffers on the Precipitin Reaction between Antiserum 125, Type III, and Homologous Soluble Specific Substance.

Dilutions of sss: 50,000; 100,000; 200,000; 400,000; 800,000; 1,600,000; 2,400,000; 3,200,000; 4,800,000. Figures in table are "greatest dilutions" in thousands.

Dilution of serum	Buffers used with final concentration and pH						Without buffer
	Citrate N/20		Phosphate M/30			Borate N/20	
	5.0		6.0	7.0	8.0	9.0	
5	1600		**1600**	**1600**			
10	1600		**1600**	**1600**	**1600**	1200	**1600**
20	1200	Post-zone	2400	(4800)	2400	1200	3200
40	800	**200**	800	(1600)	1600	800	(800)
60	(800)	**200**	<200	800	<50	(200)	800
100	1200	**400**	—	<200	—	—	<50
160	<200		—	—	—	—	—
Quotient Serum/sss	Post-zone 5000 3333 4000		Pro-zone 320,000 160,000			—	160,000

The underscored bold faced figures indicate zonal phenomena.

The action of citrate on Serum 170, Type I, is shown in Table XVI. When using this buffer a pro-zone was observed on the alkaline side. The post-zone was moved towards lower serum and higher soluble specific substance concentrations, the quotient serum/sss shifting from 2,350 to 400 for pH 6.0; in other words the ability of the soluble specific substance to keep the compound unprecipitated decreases towards alkaline reaction.

SUMMARY.

The mechanism of the precipitin reaction between antipneumococcus sera and the type-specific soluble carbohydrate is investigated. The sensitivity of the reaction is found to be generally constant when expressed by the product of the concentrations of the two reacting substances. Precipitin index (P.I.) is defined as one-millionth of the reciprocal value of this product.

TABLE XVI.

Influence of Citrate Buffers on Precipitation of Serum 170, Type I.

For precipitation without buffer see Table VII.
sss I dilutions: 1/16, 1/8, 1/4, 1/2, 1, 2, 4, 8, 16, 24, 32, 64 million.

Dilution of serum	Greatest dilution of sss showing + (or ±) reaction (For post-zone: − (or ∓) reaction)					
	pH	4.7	5.0	5.3	5.7	6.0
5		64	(64)	64	16	16
10		64	(32)	(32)	16	(24)
20		24	16	24	16	12
40		16	(16)	(12)	24	12
80		8 (1/4)	8	(8)	(8)	8
160		8 1/4	(8) (1/4)	4 1/4	4	(4)
240		<1/16	<1/16	<1/16	1 (1/8)	2 1/8
320		−	−	−	1 (1/4)	(1) 1/8
Quotient Serum/sss	Pro-zone	−	−	−	3,200,000 1,600,000 800,000	3,200,000
	Post-zone	2350 1550	1150	1550	400 600	500 400

For explanation of underscored bold faced figures see Table XI.

Zonal phenomena and their bearing on the absolute concentration and the equivalent weight of the antibody are discussed. The greater tendency towards exhibition of a post-zone in Type III is connected with the lower acid equivalent of the homologous specific carbohydrate.

The influence of the addition of normal serum and the influence of slight changes in pH are studied. The addition of normal serum as

well as the increase in pH promote the pro-zone, whereas decrease in pH promotes the post-zone. The sensitivity of the reaction is accordingly influenced by the pH in different ways depending on the range of concentration.

The precipitin index allows the recognition and elimination of zonal irregularities. Thus it offers a method for the standardization of pneumonia antibody.

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THE PRECIPITIN REACTION OF ANTIPNEUMOCOCCUS SERA.*

II. The Ratio of Precipitin to Protective Antibody

By MAE FRIEDLANDER,† HARRY SOBOTKA, Ph.D., and EDWIN J. BANZHAF, Ph.D.

(*From the Department of Bacteriology, New York University and Bellevue Medical College, and the Research Laboratory, Department of Health, New York.*)

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In the diagnosis of infectious disease precipitin and agglutinin reactions have assisted in the identification of the invading organism, when the patient's serum has been tested with suspected organisms or when antigenic material from the patient (1) has been tested against antisera.

Agglutination has been used also in the quantitative determination of the antibody in antityphoid and antimeningococcus sera. For the standardization of diphtheria antitoxin Ramon (2; see also reference 3) suggested a test-tube method based on the formation of a flocculent precipitate when toxin and antitoxin were combined in definite proportions.

The present paper records investigations concerning the parallelism of the precipitin with the protective antibody in antipneumococcus sera. The quantitative determination of antibody in antipneumococcus sera is based on the relative reactivity of the precipitin and the protective antibody; the question of their identity is not raised.

Agglutination tests in pneumonia require the use of uniform cultures; they thus share with the animal protection test the disadvantage of possible biological irregularities. Precipitin tests, however, are carried out with a reproducible non-cellular antigen of a definite chemical composition.

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† This communication is part of a thesis to be submitted by Mae Friedlander in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Bacteriology at New York University.

Method.

The precipitin indices for a number of antipneumococcus sera and antibody preparations were determined by the precipitin tests described in the preceding paper. The study of the zonal phenomena permits the elimination of these irregularities.

Only the higher values furnished by multiplication of serum and precipitinogen dilution were used in calculating the precipitin indices, all individual values smaller than one-half of the maximal value were disregarded in estimating the average of each test. Precipitin index is a millionth part of the product obtained by multiplying the dilution of the antiserum by the dilution of the antigen. For details see the preceding paper.

TABLE I.

Comparison of Precipitin and Protective Antibodies in Monovalent Type I Sera and Antibody Preparations.

No. of serum	Precipitin index	Protective units	Ratio	Reference
155	400	100	4.0	Table IX (*d*)
47	560	200	2.8	Table IX (*d*)
169	1250	200–400	4.1	Table IV
90	700	200	3.5	Table VII preceding paper
32	1720	400	4.3	Tables I and VI preceding paper
32	2550	800	3.2	Tables III and XI preceding paper
	2880†		3.6	
94	3840	800	4.8	Table IV
Preparation "f"	2340	800	2.9	Table IV
102	7170†	800	9.0	Table X preceding paper
916	1920	200	9.6	See page 88
Average ratio for the first nine values.....			3.8	

†With addition of 10 per cent normal horse serum.

The proportions of the corrected precipitin index to the protective units of the same antibody solution were compared and an unanticipated constant relation was revealed.

The quotient precipitin index/protective units for a number of monovalent Type I horses ranged between 2.8 and 4.8 with an average of 3.7. For some monovalent Type III horses the

TABLE II.

Precipitin and Protective Antibodies in Monovalent Type III Sera.

No. of serum	Precipitin index	Protective units	Ratio	Reference
125	160	4, 8, 10	16–48	Table V preceding paper
	192			Table V
126	220	4	34–55	Table V preceding paper
	135			Table V
127	128	4	32	Table V
128	85	4–10	8–21	Table V
302	575	10	58	Table X
"A"	770	10	77	Table X
"B"	6150	80	77	Table X
Average ratio of all values except the two last.....			34	

TABLE III.

Relation between Precipitin Index and Protective Potency for Polyvalent Antisera.

Horse No.	Duration of treatment	Type I			Type III		
		P. I.	P. U.	Ratio	P. I.	P. U.	Ratio
	mos.						
3079	28	720	1000	0.72	>340		
3180	28	530	500	1.06	320	10	32
			1000	0.53		(20)	(16)
3332	22	530	400	1.35	>340	10	34
			500	1.06		(20)	(17)
3335	22	350	<500	>0.70	>190	(8)	(24)
3514	11	400	100	2.0	72	2	36
			200	4.0		(4)	(18)
3530	10	480	200	2.4	256	10	26
						(20)	(13)
3531	10	60	50	1.2	>192		
3635	6	240	50	2.4	45	2	23
			100	4.8			
3640	6	60	50	1.2	30		
3639	6	<40	50	<0.8	50	2	25
Average ratio { upper 4 horses.....				0.9			24
Average ratio { lower 6 horses.....				2.3			

The parenthesized figures for protective units in Type III are based on 72 hours delay of death rather than on survival over 96 hours; on account of the pro-zone familiar in Type III pneumococcus mouse tests no survivals occurred.

quotient ranged between the extremes of 8 and 58 with an average of 34 (Tables I and II). When comparing these figures with those of Type I the almost ten times greater precipitin power associated with the protective potency in Type III is striking. If we discount this difference by referring the higher precipitin index per protective unit in Type III to the higher specific activity of equal weights of the precipitinogen rather than of the antibody, there is seen to be a similar ratio between precipitin and protective activity of the antisera for the two types investigated.

For a number of polyvalent horses[1] decidedly lower values for the quotient precipitin index/protective units were observed. In Type I they ranged between 0.7 and 2.4 with an average of 1.75 and with extreme values of 0.53 and 4.8. For Type III in polyvalent horses the quotient ranged among the lower values of those given above for monovalent sera of this type, the average being 24 (Table III).

A decrease in precipitin activity was also observed when adding one heterologous monovalent serum to an equal amount of another.

Serum 90, Type I shows a precipitin index of 700 and upon the addition of Serum 129, Type II, the precipitin index was only 60. Serum 126, Type III, decreased upon addition of No. 129 from 135 to 32.

If the Type I values of the polyvalent horses were arranged according to the duration of the treatment they could be divided into two groups; one comprising four horses under pneumococcus treatment for 2 years had quotients ranging below 1.35 with an average of 0.9, while another group of horses under treatment for no longer than 1 year had values above 1.2 with an average of 2.3.

This observation indicates that the duration of the treatment influences the proportion of the two antibody activities. This became evident from an experiment on two Type I horses whose immunization we observed from the onset. It was found that the quotients for bleedings after 37 days were about twice as high as those after 114 days; the protective potency reached greater values at a slower rate than the precipitin.

When the precipitin test was applied to refined antibody prepara-

[1] Samples of these were supplied through the kindness of Dr. Stanley Beard of the Lederle Antitoxin Laboratories, Pearl River, New York.

tions the quotients obtained were higher than from the crude sera in our experiments. We observed values as high as 9 in Type I and above 70 in Type III. There is some doubt whether this difference arises during the refining process by a selective fractionation or is simply due to a lesser proportionalism between potency and concentration in concentrated solutions.

When making due allowance for the various influences noted and

TABLE IV.

Precipitin Test of Antipneumococcus Sera, Type I, No. 94 and No. 169 and of Antibody Preparation "f" Prepared by L. D. Felton's Procedure (4).

Serum No.	Dilution of serum	Dilution of soluble specific substance in millions									Precipitin index	
		4	8	12	16	24	32	64	96	128	"P. I."	
94	20				−	−	−	−	−	−	(<320)	
					(pro-zone)							
	40				−	−	−	−	−	−	(<640)	
	80				+	+	+	±	−	−	3840	
				(post-zone)								3840
	120	−	−	−	−	−	+	−			3840	
169	20				+	+	+	+	−	−	1280	
	40				+	−	−	−	−	−	640	
	80				−	−	−	−	−	−	<1280	1250
	120	+	+	+	−	−	−	−			1440	
	160	+	+	+	−	−	−				1920	
	240	−	−	−	−	−	−				(< 960)	
"f"	20				+		+	±	±	+	2560	
	40				+		+	+	−	−	2560	2340
	60				+		±	±	−	−	1920	
	120				−		−	−	−	−	(<1920)	

discussed in the present and in the preceding paper a fairly accurate idea of the amount of antibody contained in an antipneumococcus serum can be obtained. The determination of the precipitin index may replace the slow mouse test in the course of immunizing horses as well as during refining processes; for final controls animal tests will have to be retained.

EXPERIMENTAL PART.

In the following tables data are collected for several monovalent Type I and Type III sera. Some of them like No. 94, Type I, Table IV, and No. 125 and No. 127, Type III, Table V, show a pro-zone and

TABLE V.

Precipitin Test of Antisera 125 to 128, Type III.

Dilution of serum	Dilution of soluble specific substance III in millions															P. I.
	0.05	0.1	0.2	0.3	0.4	0.6	0.8	1.2	1.6	2.4	3.2	4.8	6.4	9.6	12.8	
No. 125																
10	+	+	+	+	+	+	+	+	+	−	−	−				(16)
20	+	+	+	+	+	+	+	+	+	±	−	−				(40)
40	−	+	+	+	+	+	+	+	+	+	+	+				192
80		±	−	+	−	−	−									(24)
120		−	−	−	−	−	−									−
No. 126																
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	112
20	+	+	+	+	+	+	+	+	+	+	+	+	+	±	−	160
40	±	+	+	+	+	+	+	+	±	±	−	−				(64)
80		−	−	−	+	±	−									(40)
120		+	−	−	±	−	−									(42)
No. 127																
10	+	+	+	+	+	+	+	+	+	−	−	−				(16)
20	+	+	+	+	+	+	+	+	+	−	−	−				(32)
40	+	+	+	+	+	+	+	+	+	+	+	−				128
80		+	+	+	−	+	−									(48)
120		−	−	−	−	−	−									−
No. 128																
10	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	64
20	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	96
40	±	−	+	+	+	+	+	+	+	+	−	−	−	−	−	96
80		+	±	−	−	±	−									(40)
120		±	+	+	±	+	−									72

in these instances only the high values obtained from higher serum dilutions were taken into consideration. Values lower than 50 per cent of the highest value in a test are parenthesized and were disregarded.

In Table VI experimental results for Type I and Type III of ten

polyvalent horses, immunized against three types, are given. The figures are the dilution products or precipitin indices for the individual serum dilutions given in the first column. Pro-zones of more or less extent, may be observed in Sera 3079, 3180, 3640 and others for both types, in Sera 3514 and 3530 for Type I, and almost in all sera for serum dilution 1:5 for Type III.

TABLE VI.

Synopsis of Precipitin Tests of Sera Obtained from Ten Polyvalent Horses (Types I, II, III).

Precipitin indices for single serum dilutions given. Figures in parenthesis disregarded in computation of average P.I.

Dilution of serum	Horse No.									
	3079	3180†	3332	3335†	3514	3530	3531	3635	3640	3639
	Type I									
5	(80)	(40)	—	320	(30)	(20)	40	(120)	(<20)	Less
10	(80)	(80)	—	320	(120)	(60)	60	(80)	(<20)	than
20	(80)	(160)	(240)	320	(160)	(160)	40	(40)	60	40
40	(160)	320	320	320	(160)	(160)	80	(80)	60	
80	480	640	640	320	320	320	(<40)	240	(<40)	
160	960	640	640	480	480	640	(<80)	240	(<80)	
	Type III								‡	‡
5	(64)	(>128)	—	>128	—	>128	>128	32	(8)	32
10	(64)	(96)	—	192	—	>256	>256	32	32	48
20	(48)	(128)	—	>256	64	256	192	64	32	64
40	256	256	256	256	64	384	(64)	(<24)	28	28
80	>256	192	>256	128	64	256	(128)	(<16)	(<16)	16
160	>512	>512	>512	(96)	96	(96)	(<32)	—	—	(<32)

† see Table VII. ‡ see Table VIII.

Examples for both regular and pro-zone sera of each type are given in Tables VII and VIII.

A synopsis of these values is given in Table III, page 81, where the precipitin indices as calculated from Table VI are listed in the third and sixth columns. The protective units in the fourth and seventh columns of Table III, and in the third columns of Tables I and II were obtained from mouse tests, carried out according to the government standards in the Research Laboratories, Department of Health, City of New

TABLE VII.

Reaction of Soluble Specific Substance Type I with Polyvalent Antipneumococcus Sera.

Serum 3335 (Very Regular Test).

Dilution of serum	Dilution of soluble specific substance in millions								
	0.5	1	2	4	8	16	32	64	128
5				+	+	+	+	+	−
10				+	+	+	+	−	−
20			+	+	+	+	−	−	
40			+	+	+	−	−	−	
80	+	+	+	+	−	−			
120	+	+	+	±	−	−			
Serum 3180 (Pro-Zone Type).									
5				+	+	−	−	−	−
10				+	+	−	−	−	−
20			+	+	+	−	−	−	
40			+	+	+	−	−	−	
80		+	+	+	+	−	−		
120		+	+	+	−	−	−		

TABLE VIII.

Reaction of Soluble Specific Substance Type III with Polyvalent Antipneumococcus Sera.

Serum 3639 (Regular).

Dilution of serum	Dilution of soluble specific substance in millions							
	0.2	0.4	0.6	0.8	1.6	3.2	6.4	12.8
5				+	+	+	+	−
10				+	+	+	±	−
20			+	+	+	+	−	
40			+	±	−	−	−	
80	+	−	−	−	−			
120	−	−	−	−	−			
Serum 3640 (Pro-Zone).								
5				+	+	−	−	−
10				+	+	+	−	−
20			+	+	+	−	−	
40			+	±	−	−	−	
80	−	−	−	−	−			
120	−	−	−	−	−			

TABLE IX.

Development of Precipitin and Protective Antibodies against Type I Diplococcus Pneumoniæ in Two Horses.

Dilution of serum	Precipitin indices for individual serum dilutions. Duration of immunizing treatment.							
	(a) 0		(b) 37		(c) 72		(d) 114 days	
	47	155	47	155	47	155	47	155
10			(60)	(120)	(<40)	(<40)	(30)	(160)
20			(80)	160	(80)	(40)	(40)	(60)
40	Nil	Nil	(120)	240	(160)	(80)	(240)	(80)
80			640	320	480	320	640	320
160			640	(<160)	480	640	480	480
Average P.I.			640	240	480	480	560	400
Protective units	Nil	Nil	80–100	40	80	100	200	100
Ratio	—		6.4–8.0	6.0	6.0	4.8	2.8	4.0

TABLE X.

Precipitin Test of Serum 302, Type III, and of Two Globulin Fractions A and B, Concentrated 16 Times and 14 Times Compared with the Original Serum 302.

Dilution of antibody solution	Dilution of precipitinogen in million												P. I.
	0.2	0.4	0.8	1.2	1.6	3.2	4.8	6.4	9.6	12.8	25.6	51.2	
No. 302													
20	+	+	+	+	+	−	−	−	−	−	−		(32)
40	+	+	+	+	+	+	−	−	−	−	−		(128)
80	+	+	+	+	+	+	+	+	−	−	−		512
160	+	+	+	+	+	+	±	−	−	−	−		640
320	−	−	−	−	−	−	−	−	−	−	−		−
A													
80	+	+	+	+	+	+	+	−	−	−	−		(384)
160	+	+	+	+	+	+	−	−	−	−	−		(512)
320	+	+	+	+	+	+	−	−	−	−	−		1024
480	−	−	−	−	+	−	−	−	−	−	−		768
B													
160	+	+	+	+	+	+	+	+	+	+	+	±	6144
320	−	−	−	+	+	+	+	−	−	−	−	−	(1536)
480	+	+	−	−	+	+	−	−	−	−	−	−	(1536)
A+B													
160	+	+	+	+	+	+	+	+	+	+	+	±	Stronger reactions than B alone

York, by Miss G. M. Cooper under the direction of Dr. W. H. Park, and in the Testing Department of the Lederle Antitoxin Laboratories, Pearl River, New York, by Miss F. L. Clapp, to whom we have to express our thanks for their kind cooperation.

The influence of the duration of the treatment on the quotient precipitin index/protective power was studied in two monovalent horses, No. 47 and No.155, for which the experimental data are to be found in Table IX. The quotients were 6 to 8 after 5 weeks, 4.8 to 6.0 after 10 weeks and 2.8 to 4.0 after 16 weeks.[2]

Two globulin fractions were obtained by the ammonium sulfate method (6) from Serum 302 (Table X). The first was concentrated to one-sixteenth, the second to one-fourteenth of the original volume. The first fraction contained only ten mouse units per cc. like the starting material, in the second a concentration of 80 units was attained. The ratio precipitin/protection, already as high as 58 in the original serum, rose to 77 for the globulins. By mixing the two globulin fractions in equal amounts an effect was observed similar to those discussed on page 65 of the preceding paper.

The precipitin values for Preparation 916, Type I, 200 protective units (Table I), were

for a serum dilution of	10	1280
	20	2560
	40	160
	80	80
	160	160;

in this preparation, as in No. 102 of Table VI in the preceding paper, the precipitin activity was very high as compared with the protective potency.

SUMMARY.

The precipitin indices for a number of monovalent and polyvalent antipneumococcus sera were determined under known conditions, and found to vary as did the number of protective units.

The ratio precipitin index/protective units in monovalent sera was

[2] Forster (5), investigating the relation between the time elapsed since immunization and the precipitin titer, observed the development of a post-zone during the "secondary stage."

found to lie between 2.8 and 4.8 for Type I and to be about ten times greater for Type III.

Lower values were found in polyvalent horses and when mixing heterologous monovalent sera with each other.

The influence of the duration of treatment upon the quotient was studied.

Several refined and concentrated preparations showed a relative increase in precipitin activity.

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THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

I. Demonstration of a Type-Specific Substance in Extracts of Streptococcus hæmolyticus.

By REBECCA C. LANCEFIELD, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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Chemical and immunological studies to determine the nature of the substances responsible for the specific reactions of a number of microorganisms have been reported recently by several investigators. Most of this work has been reviewed in some detail elsewhere (1). In certain very carefully studied instances, such as pneumococcus (2) and the Friedländer bacillus (3), specificity is determined by type-specific carbohydrates, while non-specific group reactions are due to species-specific proteins. The same general relationship holds for the *Streptococcus viridans* (4).

Hitchcock (5) found that nearly all hemolytic streptococci yielded in crude antiformin extracts a "residue antigen," such as described by Zinsser and Parker (6), which reacted with all of his antibacterial sera prepared against hemolytic streptococci but not with sera against green streptococci. Conversely (7) similarly prepared "residue antigens" from non-hemolytic streptococci did not react with his hemolytic streptococcus antisera.

Hirsch (8) also reported finding a precipitating substance in extracts of hemolytic streptococci which he called the soluble specific substance. He did not give any evidence to support this statement other than the fact that it precipitated antibacterial sera and sera prepared against solutions of this material. He did not state whether his sera and antigens were all prepared and tested with one or with several strains.

In repeating the work of Hitchcock as a preliminary to the present experiments, it was found, indeed, that all partially purified antiformin extracts, or extracts prepared in such a way as to remove the bulk of the protein, precipitated equally well antibacterial sera made against

different types of hemolytic streptococci. The precipitates formed typical discs like those obtained with type-specific carbohydrates in other species of bacteria. All attempts to separate a type-specific fraction from such extracts by fractional alcoholic precipitations were unsuccessful. Methyl and ethyl alcoholic extracts of whole bacteria contained the same species-specific substance but no trace of a type-specific fraction.

It was then found that extracts made by Porges' method (9) for the removal of bacterial capsules contained a type-specific fraction. This report is the presentation of some of the work carried out with this fraction.

The strains used were chiefly those which had been previously grouped by agglutination and protection tests (10). These had been preserved for 4 or 5 years by dessicating them while in the frozen state by Swift's (11) technique. Cultures recovered from this stock, as a rule, retained the virulence and cultural and serological behavior characteristic of them at the time of dessication. These old strains were used in preference to freshly isolated ones because the distinctive types had already been separated and grouped. The results obtained by any new method of grouping must, of necessity, agree with those obtained by previous methods in order to establish its validity.

Methods.

Antigens were prepared from the various strains by a modification of Porges' method as follows: bacteria centrifuged from 18 hour plain broth cultures, were suspended in 0.85 per cent NaCl solution to which sufficient N/1 HCl was added to make a final concentration of N/20 HCl. Usually the sediment from 1 liter of broth culture was extracted in 10 cc. to 15 cc. volume. The suspension in a Pyrex centrifuge tube was immersed in boiling water for 15 minutes with occasional stirring, then cooled, centrifuged, and the supernatant fluid removed and neutralized with N/1 NaOH. The precipitate which appeared on neutralization was thrown down in the centrifuge; and the water-clear, slightly yellowish supernatant fluid was used as antigen for precipitin tests. When a more potent antigen was desired, it was obtained by extracting in smaller volume or by concentrating the dilute extract described above. For concentration, crystals of sodium acetate—usually 10 gm. per liter of extract—were added to facilitate the subsequent precipitation with three or four volumes of 95 per cent alcohol. After standing overnight in the ice box, the alcoholic precipitate was thrown down and redissolved in salt solution. The greater part of the alcoholic precipitate was insoluble; but

precipitin tests showed that very little loss of active material was occasioned by discarding the insoluble part. The alcoholic precipitation was repeated and the

TABLE I.

Precipitin Reactions with Unabsorbed Antibacterial Serum from Rabbit Q867, Immunized with Strain S60, Type S60.

Antigen: HCl extract			Read after 2 hrs. at 37°C.	Read after additional 18 hrs. in ice box
Strain	Type	Cc.		
S60	S60	0.4	+++	+++
		0.3	+++	+++
		0.1	++	++
S43	"	0.4	++	+++
		0.3	+++	+++±
		0.1	++	++
S23	S23	0.4	−	±
		0.3	−	+
		0.1	±	++
S65	"	0.4	−	+
		0.3	−	+±
		0.1	±	++
S3	S3	0.4	−	±
		0.3	−	+
		0.1	±	++
S144	"	0.4	−	±
		0.3	−	+
		0.1	±	++
S24	Unclassified	0.4	−	±
		0.3	−	+±
		0.1	±	++
S276	"	0.4	−	±
		0.3	−	+
		0.1	±	++

In all tables ±, +, ++, +++, ++++, indicate degrees of reaction; − indicates a negative reaction; 0 indicates that the test was not made.

antigen concentrated to any desired volume by this means. In the following experiments, most of the antigens were the original extracts, although a few were concentrated by the alcoholic precipitation method.

Antibacterial sera were prepared by inoculating rabbits intravenously with increasing doses of heat-killed broth cultures followed by living organisms. After 6 or 8 weeks of immunization, the sera of such animals contained agglutinins and precipitins and, in some instances, protective antibodies for the homologous strain. Precipitins for antigens from strains of unlike type were also present in most of these sera. Experiment 1 gives the typical precipitin reactions of the kind of antibacterial serum which gave only moderate cross-reactions with heterologous antigens.

TABLE II.

Precipitin Reactions with Unabsorbed Antibacterial Serum from Rabbit Q609, Immunized with Strain S23, Type S23.

Antigen: HCl extract			Read after 2 hrs. at 37°C.	Read after additional 18 hrs. in ice box
Strain	Type	Cc.		
S23	S23	0.4	++++	++++
		0.1	+++	+++
S65	"	0.4	+++	+++
		0.1	++	++
S60	S60	0.4	+	++
		0.1	+±	++
S128	"	0.4	+±	++
		0.1	++	++±
S3	S3	0.4	−	±
		0.1	+	++
S144	"	0.4	−	±
		0.1	+	++
S24	Unclassified	0.4	++±	++±
		0.1	+++	+++
S276	"	0.4	±	++
		0.1	+	+±

Experiment 1.—Eight HCl extracts were made from two strains of each of three types of hemolytic streptococcus and from two unclassified strains. Three dilutions of each antigen were set up as follows: 0.4 cc., 0.3 cc., and 0.1 cc. were placed in successive tubes, and the volumes made up to 0.4 cc. with salt solution. 0.1 cc. of serum from Rabbit Q867, immunized as indicated in Table I, was added to each tube; and, after mixing, the tubes were incubated in a 37°C. water bath for 2 hours. The tests were read immediately, and again after standing overnight in the ice box. Controls of serum alone, of antigens alone, and of antigens with normal serum were negative.

Table I shows that antigens of the type homologous to the serum formed heavy flocculent precipitates almost as soon as they were mixed with the serum. The maximum reaction with these antigens was reached during the initial incubation at 37°C., and the intensity changed very little during the ensuing period in the ice box. Heterologous antigens, on the contrary, precipitated the serum slowly, often forming no visible precipitate during the 2 hour period in the water bath, but, after standing overnight in the ice box, they gave disc precipitates similar to those formed by specific carbohydrates of some species of bacteria. The optimum zone for disc precipitates was usually in considerably higher dilutions than for the more nearly type-specific flocculent precipitates.

Experiment 2.—A similar experiment was performed with a slightly different series of antigens and antibacterial serum from another rabbit, immunized as shown in Table II. The same series of tests was set up as in Experiment 1, except that the tube containing 0.3 cc. of antigen was omitted.

The reactions shown in Table II were typical of another kind of antibacterial serum with which immediate pronounced cross-reactions were obtained. Considerably less specificity was evident in the precipitin reactions in this instance than in Experiment 1, in which the 2 hour reading seemed quite type-specific; but, in Experiment 2, this reading showed a large amount of cross-reaction. On the basis of this test the classification of Strains S60 and S128 was doubtful; and Strain S24 would have been placed in *Type* S23 if other evidence had not been available. No conclusion could be drawn from the character of the precipitate in these instances, since it had much the same appearance for the heterologous antigens as for the homologous. Some other means of distinction was necessary.

Accordingly, isolation of the type-specific antigen was attempted but was only partly successful. Fractional alcoholic precipitations served to separate the non-type-specific disc-forming substance; but no satisfactory method was devised for removing the substance which gave non-type-specific flocculent precipitates with immune serum; hence attempts were made to prepare type-specific antisera by absorption. Table III shows such an experiment.

Experiment 3.—Serum from Rabbit Q309, immunized as shown in Table III, was absorbed with a heterologous hemolytic streptococcus. The bacteria from 1.5 liters of plain broth culture of a heterologous strain were centrifuged, resuspended in a small volume of salt solution, and killed by heating at 56°C. for 1 hour. To the packed bacteria, 2 cc. of immune serum diluted with 4 cc. of salt solution were added. A parallel absorption was performed at the same time with bacteria from the strain homologous to the serum. Controls of immune serum and of normal serum similarly diluted were included, and the absorption carried out at 37°C. for half an hour. After centrifugation the clear supernatant diluted serum was removed and preliminary precipitin tests made. Since precipitates were no longer obtained with heterologous antigens, the absorption was considered complete. Table III shows the precipitin tests with a number of homologous and

TABLE III.

Absorption Experiment: Precipitin Reactions with Serum from Rabbit Q309, Immunized with Strain S23, Type S23.

Antigen: HCl extract		Not absorbed	Absorbed with heterologous Strain S60
Strain	Type		
S23	S23	+++	+++±
S65	"	++±	++
S60	S60	+	−
S43	"	++	−
S128	"	++	−
S4	"	++	−
S24	Unclassified	++	−
S276	"	+	−
R28	"	++	−

heterologous HCl antigens, and the heterologous absorbed serum, also the control unabsorbed serum. 0.2 cc. of serum dilution (equivalent to 0.07 cc. of undiluted serum), and 0.2 cc. of antigen were mixed, and incubated in a 37°C. water bath for 2 hours. Readings were made after an additional 18 hours in the ice box. All necessary controls were negative.

Table III shows that the serum absorbed with a heterologous strain had become, in effect, a type-specific serum. This absorbed serum reacted only with antigens of the type used in immunization, while reactions with heterologous antigens were all completely negative. The heterologous strain had, therefore, not only absorbed the anti-

bodies for all strains of its own type but also the antibodies for other heterologous strains. The control lot of unabsorbed serum gave good reactions with most of these antigens. Absorption of the same serum with the homologous strain removed the antibodies for the homologous antigen also. In view of these results it was evident that Strain S24 did not belong to *Type* S23, as might have been supposed from

TABLE IV.

Absorption Experiment: Precipitin Reactions with Serum from Rabbit Q612, Immunized with Strain S23, Type S23.

Antigen: HCl extract		Not absorbed	Absorbed with hemolytic streptococcus of				
			Homologous type:	Heterologous type:			
Strain	Type		S65, Type S23	S128, Type S60	S144, Type S3	S24, unclassified	New York 5, scarlatinal
S23	S23	++±	−	++	++	++±	++±
S65	"	+++	−	+++	+++	++	++
S39	"	+++	−	++±	+++	+++	+++
S60	S60	++	−	−	−	−	−
S6	"	+±	−	−	−	−	−
S128	"	++	−	−	−	0	0
S4	"	++	−	−	−	−	±
S43	"	++	−	−	−	−	−
S72	"	++	−	−	−	0	0
S3	S3	+	−	−	−	−	−
S80	"	+	−	−	−	−	−
S144	"	+	−	−	−	−	−
S149	"	+	−	−	−	−	−
S24	Unclassified	++±	−	−	−	−	−
S276	"	±	−	−	−	0	0

the heavy precipitation with unabsorbed serum from Rabbit Q609 (Table II).

Numerous similar absorption experiments were performed with different sera and with different hemolytic streptococci as absorbing agents; essentially similar results were obtained in all these experiments. Titration of absorbed serum with varying dilutions of heterol-

ogous antigens showed that shifting of the prozone was not responsible for the negative results. Finally, a more comprehensive experiment was performed.

Experiment 4.—Five aliquot portions of serum from Rabbit Q612 were absorbed respectively with five different strains of *Streptococcus hæmolyticus*, and a sixth portion kept as an unabsorbed control. Only one strain was the type homologous

TABLE V.

Absorption Experiment: Precipitin Reactions with Serum from Rabbit Q867, Immunized with Strain S60, Type S60.

Antigen: HCl extract		Not absorbed	Absorbed with hemolytic streptococcus of				
Strain	Type		Homologous type:	Heterologous type:			
			S128, Type S60	S65, Type S23	S144, Type S3	S24, unclassified	New York 5, scarlatinal
S23	S23	+	−	−	−	−	−
S65	"	+±	−	−	−	−	−
S39	"	+	−	−	−	−	−
S60	S60	++±	−	++±	++±	++	+++
S6	"	++	±	++	++	++	++±
S128	"	++	−	−	+	0	+
S4	"	++	−	+	+±	+±	++
S43	"	+++	−	++	++	++	++±
S72	"	++±	−	+	++	0	++
S3	S3	±	−	−	−	−	±
S80	"	+	−	−	−	−	±
S144	"	+±	−	−	−	−	−
S149	"	+	−	−	−	−	−
S24	Unclassified	+	−	−	−	−	±
S276	"	+±	−	−	−	0	−

to the serum; three others were known heterologous types; the fifth belonged to an unclassified group. Absorption was accomplished with one or two 1 hour incubations, and was proved to be complete by preliminary testing with HCl extracts from heterologous strains. After being completely absorbed these sera were tested with HCl extracts from a number or strains representing different types (see Table IV). Readings with the absorbed sera made after 2 hours incubation at 37°C. and 18 hours in the ice box agreed. Controls of serum and antigen alone, and of antigen with normal serum, were negative.

A similar absorption experiment was performed with serum from Rabbit Q867, immunized with another type (see Table V).

This experiment showed again that unabsorbed antibacterial sera reacted with most antigens made from heterologous strains, although they reacted more strongly with homologous antigens than with heterologous. Absorption with bacteria of the homologous strain, or with bacteria of any strain of the same type, removed all antibodies; while absorption with any heterologous strain removed antibodies for *all* types of heterologous hemolytic streptococci, but left the type-specific antibodies practically intact. By this method, therefore, it was possible to prepare a serum which contained only type-specific antibodies.

Certain technical difficulties were encountered in these absorption experiments: complete absorption was often hard to attain because the serum could not be much diluted if it were to be used subsequently for satisfactory precipitin tests. Non-type-specific antibodies were absorbed more readily by certain strains than by others. Strains of the homologous type were more efficient in this respect than heterologous strains; and heterologous strains varied somewhat among themselves. Of the heterologous strains used in these experiments, S24 was the most effective absorbing agent. Various other workers have observed that some strains of bacteria are better than others for absorbing antibodies; and Krumwiede, Cooper, and Prevost (12) point out, in their comprehensive paper on agglutinin absorption, that this is often true. The present experiments with hemolytic streptococci indicate also that few absorptions with heavy emulsions are preferable to often repeated ones with fewer organisms. This is due partly to unknown factors, but can be partially explained by the additional dilution occasioned by using wet bacteria and also by the prolonged heating involved in repeated absorptions. Numerous absorptions with heterologous strains also tend eventually to reduce somewhat the titer of the type-specific antibodies. This may be due to a non-specific adsorption, such as occurs with kaolin, rather than to a lack of immunological specificity in the absorption process.

Examples of such overabsorption were usually found in sera which contained large amounts of non-type-specific antibody and conse-

quently required excessive absorption for its removal. Even such repeatedly absorbed sera still reacted, though in less degree, with most homologous antigens and not at all with heterologous. Such instances emphasize the importance of selecting sera with as little non-type-specific antibody as possible as well as the necessity of proper selection of the absorbing strain.

From three strains it was impossible to obtain a type-specific fraction by any of the methods which were usually successful. In one instance this was associated with loss of agglutinability; although in two others the strains were still agglutinable. While these were all old laboratory strains, so also were most of the other strains used in these experiments. It is possible that certain strains never possessed the function of producing a type-specific antigen; but it seems more probable, especially in view of the earlier successful classification of these strains, that some such factors as length of time under cultivation caused this condition. A study of other known groups of hemolytic streptococci might furnish a solution of this question.

DISCUSSION.

A type-specific substance was detected in HCl extracts of hemolytic streptococci by the absorption method. Such extracts also contained non-type-specific substances which gave confusing cross-reactions in the precipitin test when unabsorbed immune serum was used. Homologous antigens usually formed heavy flocculent precipitates as soon as they were mixed with the serum; while heterologous antigens often formed no precipitate until after the tubes had been in the ice box overnight. These non-type-specific precipitates were discs like those characteristic of type-specific carbohydrates of other species. Occasionally, however, non-type-specific precipitates appeared early and had the same flocculent characteristics as those formed by homologous antigens. Such results precluded the possibility of obtaining reliable type-specific precipitin reactions unless either the non-type-specific substances could be removed from the antigen or the non-type-specific antibodies from the serum.

While complete purification of the antigen by fractional precipitation proved extremely difficult or impossible, the preparation of type-specific serum by absorption was found to be easy. Although absorp-

tion with any strain of the homologous type removed all antibodies from the serum, absorption with bacteria from heterologous strains of hemolytic streptococci removed only the non-type-specific antibodies with the result that the serum no longer gave cross-precipitations with extracts from any heterologous strain of hemolytic streptococcus but still reacted with extracts from homologous strains, usually with only slight change in intensity. Numerous absorptions with heterologous bacteria eventually reduced the titer of type-specific antibodies; but it is probable that this is a non-specific adsorption, in the category of adsorption of antibodies by substances like kaolin, rather than an invalidation of the other experiments reported. Even in these extreme instances, however, type-specific reactions were still obtained with the absorbed serum. It was possible, therefore, to prepare antibacterial sera which contained only type-specific antibodies and consequently gave only type-specific precipitin reactions.

Of nineteen strains of hemolytic streptococcus studied, ten yielded type-specific antigens, six were tested for non-type specific antigens only since no homologous serum was available in these instances, and three failed to produce type-specific antigens. It seems probable that this failure was associated with long cultivation in the laboratory, or with other unknown factors, since these strains had been classified several years before by other methods. The classification of all other strains by the method described here agreed with that previously determined for these strains by agglutination and protection tests.

Obviously this has distinct advantages for classification of hemolytic streptococci over that possessed by the agglutination reaction. So many strains of hemolytic streptococci agglutinate spontaneously that the only methods previously applicable to their grouping were agglutinin absorption or serum protection of animals with which they were inoculated. Both methods are costly in time and material. Moreover, protection tests with strains of low virulence are impossible or inconclusive. The complexity of the antigenic structure of certain hemolytic streptococci makes the interpretation of results of agglutinin absorption at times very difficult, as shown recently by several authors (13). The application of this method to such groups might reveal a type-specific element in these strains, whereas the agglutinin absorption method has failed to indicate sharply defined types but has led to the suggestion by some of these authors of an antigenic mosaic.

The precipitin test, in contrast with the agglutination and protection tests, is applicable to any strain. The HCl extract can be prepared directly from the sediment of broth cultures in a few hours; and even when requiring additional concentration this can be completed in a day or two. The absorption of the non-type-specific antibody from the serum is the only time-consuming part of the method. Once absorption is complete, however, the sera may be kept in the ice box for months and used as required. The facility with which certain strains absorb non-type-specific antibodies is noteworthy and makes desirable the conservation of these types for this special purpose.

SUMMARY.

1. Hydrochloric acid extracts of *Streptococcus hæmolyticus* contain type-specific, as well as non-type-specific, substances. The precipitates formed by these crude extracts with homologous antibacterial serum are flocculent, while those obtained with heterologous serum are usually disc-like.

2. The type-specific substance may be detected by the use of antibacterial sera absorbed with heterologous strains of hemolytic streptococci. Such absorbed sera are type-specific: they are precipitated only by extracts of strains of the homologous type.

3. Any heterologous strain of hemolytic streptococcus absorbs the antibodies for all other heterologous strains, but homologous strains absorb type-specific antibodies as well. Numerous repeated absorptions with heterologous hemolytic streptococci tend to lower the titer of the type-specific antibody. A possible explanation of this fact is suggested.

4. Three strains did not yield a type-specific substance; and it seems probable that they had lost this function because of long continued cultivation in artificial media.

5. Classification based on the precipitin test with absorbed serum agrees with that previously determined by agglutination and protection tests. The method is, therefore, applicable to the problem of classification of the hemolytic streptococci.

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CHANGES IN THE PERCENTAGE OF CALCIUM AND PHOSPHORUS OF THE BLOOD FOLLOWING SECTION OF THE SYMPATHETIC AND VAGUS NERVES.*

By BENJAMIN N. BERG, M.D., ALFRED F. HESS, M.D., and ELIZABETH SHERMAN.

(*From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York.*)

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That calcium and potassium, as well as other ions, are capable of stimulating or depressing the activity of nerves has been known for many years, having been established by the brilliant studies of Jacques Loeb as well as others. Recently the conception of the interaction between nerves and inorganic salts of the body has been amplified so as to include a reciprocal relationship between the two—not only an effect of ions on the function of the nerves, but of nerve impulses on the distribution of various ions.

The latter conception has been emphasized especially by the recent work of Kraus and Zondek (2) who suggested that the nerves exert their specific functions by means of the various ions, that, for example, the sympathetic system has an action similar to calcium or rather to an artificial excess of calcium. Elaborating this idea somewhat further Zondek (3) stated that the ions are the very materials made use of by the nerves to fulfill their functions, that stimulation of the vagus leads to a preponderance of potassium, whereas similar stimulation of the sympathetic leads in turn to the preponderance of calcium. The nerves are supposed to control not only the distribution of these ions within the cells and cell membranes, but the hydrogen ion balance as well. At about the same time Billigheimer (4) brought forward a similar point of view and showed that stimulation of the sympathetic nerves by means of suprarenin induced lowering of the calcium level of the blood, a result which was interpreted as due to a local increase of calcium within the area of stimulation. Pilocarpin is stated to have had the opposite effect in relation to calcium, but in this regard the interpretation seems questionable.

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* Presented at the Annual Meeting of the American Society of Biological Chemists, Rochester, April 14, 1927 (1).

In association with studies of the calcium and phosphorus metabolism in rickets and tetany, it seemed of interest to determine whether the concentration of these ions would be appreciably affected by lesions of the sympathetic and parasympathetic systems. Accordingly, a series of experiments were carried out in which the great abdominal centers of the sympathetic system were largely destroyed or the vagi were severed within the abdomen. The percentage of calcium and inorganic phosphate of the blood was then closely followed for prolonged periods. In this connection it may be mentioned that in 1867 Eckhard (5) reported that by cutting the splanchnic nerves he was able to bring about the appearance of sugar in the urine, a manifestation which was, however, not constant and which he attributed to a stimulative effect. Recently Nakao (6) sectioned the splanchnic nerves in rabbits in order to study the effect on the excretion of urine; he concluded that this lesion decreased diuresis and lessened the percentage of chlorides in the blood.

Operative Procedure.

All of the operations were performed on dogs under complete ether narcosis. Exposure of the nerves was accomplished by means of an upper median abdominal incision, except in relation to the left splanchnic which in some instances was exposed by the lumbar route. At every operation, in addition to division of the nerve trunks, traction was made and a segment 2 to 3 cm. in length was resected in order to preclude the possibility of regeneration. The sympathetic ganglia and plexuses likewise were resected and were examined histologically for verification of the tissue. The operations were performed with a minimum degree of exposure and handling of the viscera.

Operations on the Splanchnic Nerves.—The right splanchnic was exposed solely by the intraperitoneal route. The left splanchnic was exposed either intraperitoneally or extraperitoneally. These nerves were isolated below the internal arcuate ligaments of the diaphragm. In order to identify them, it was found of value to follow cephalad the branches to the suprarenal glands and then to expose the main trunk by traction.

Operations on the Celiac and Superior Mesenteric Plexuses and Ganglia.—The method which was found most satisfactory for the removal of these structures was preliminary isolation of the hepatic and celiac arteries, and separation of the right celiac ganglion. The dissection was followed to the aorta and continued caudad along its ventral surface and to the left of the vena cava, extending to the superior mesenteric artery, so that the superior mesenteric and left celiac ganglia were also separated. All of the intervening tissue lying between the suprarenals

was excised, care being taken to avoid injury to the glands. Careful dissection of the retroperitoneal tissues was necessary in order to avoid the receptaculum chyli and the larger lacteals. As large an area as possible was included in the resection of the plexuses and ganglia, which were removed *en masse*. The amount removed varied from case to case.

Experiments on the Abdominal Sympathetics.

A considerable number of operations of this kind were carried out. The results are summarized in Table IV. Characteristic data of

TABLE I.

Calcium and Inorganic Phosphate of the Blood Following Partial Removal of the Celiac Plexus.

Dog 3	Date	Interval after operation	Blood		Remarks
			Inorganic P	Serum Ca	
	1926		*mg.*	*mg.*	
Mongrel	12/8		2.87	10.7	Previous to operation
♀		3 hrs.	3.79	9.1	Diet: bread and cooked meat
9 kg.	12/9	25 "	3.87	7.5	
		25¼ "	2.47	6.4	Injected intravenously 0.1 mg. epinephrine (in 1 cc. 0.85 per cent NaCl)
	12/10	49 "	3.26	6.8	
	12/11	68 "		6.9	
	12/13	5 days	2.76	5.9	Temperature 38.5°. Blood CO_2 58.8
	12/14				
	12/16	8 "	3.69	6.8	Blood sugar 85.1 mg.
	12/21	13 "	4.27	6.4	" " 92.7 "
	12/23	15 "	3.16	6.7	" " 93.4 "
	1927				
	1/4	27 "	4.09	11.2	Intermittent fever and nasal discharge
	1/5				Weight 9 kg. Killed. Diffuse bronchopneumonia

operations in which the celiac plexus was partially removed, or the right or left splanchnic nerves divided, are reproduced in Tables I, II, and III. A review of these tables shows that the calcium level of the serum fell consistently following destruction of the celiac plexus or of the splanchnic nerves. In the first case which is charted, the percentage reached the low level of 5.9 mg. It is of in-

terest to note that in spite of this low level of calcium, the animal evinced no signs of tetany. The Kramer-Tisdall method was used for

TABLE II.

Calcium and Inorganic Phosphate of the Blood Following Division of Right Splanchnic Nerve.

	Date	Interval after operation	Blood		Remarks
			Inorganic P	Serum Ca	
Dog 6	*1927*		*mg.*	*mg.*	
Mongrel	1/4		2.63	11.0	Previous to operation
♀		2 hrs.	5.58	8.2	Diet was bread and cooked meat
6 kg.	1/5	25 "	3.02	6.0	
	1/11	7 days	4.28	7.8	
	1/21	17 "		10.35	Galvanic reactions: cathodal closing 2.5–3.0, opening 10.5
Dog 7					
♂	1/11		2.03	10.0	Previous to operation*
		2½ hrs.	2.27	6.9	Diet was bread and cooked meat
	1/12	24 "	2.35	7.3	
	1/21	9 days		8.3	
	1/22				Galvanic reactions: cathodal closing 1.9, opening 9.5
	1/31	19 "	2.10	10.2	

* 2 cm. of the nerve was resected.

TABLE III.

Calcium and Inorganic Phosphate of the Blood Following Division of Left Splanchnic Nerve.

Dog 9	Date	Interval after operation	Blood		Remarks
			Inorganic P	Serum Ca	
	1927		*mg.*	*mg.*	
♂	1/24		2.51	9.8	Previous to operation
8 kg.	1/25	2½ hrs.	2.75	7.5	
	1/26	24 "	2.91	8.1	
	1/31	6 days		10.0	

estimating calcium and the Briggs modification of the Bell-Doisy method for estimating the inorganic phosphorus. We did not at-

tempt to ascertain what ratio of the total calcium was diffusible or in an ionized state. To bring about this marked reduction of calcium, it sufficed to sever but one of the splanchnic nerves, and it did not seem to be of consequence whether this was the right or the left. The reduction in the calcium level occurred in about 24 hours, but the percentage was still low in some instances after an interval of 1 or 2 weeks. In all cases, however, it regained its normal level, evidently due to the action of a compensatory mechanism. The accompanying graph gives a typical picture of the course of the serum calcium following the severing of a splanchnic

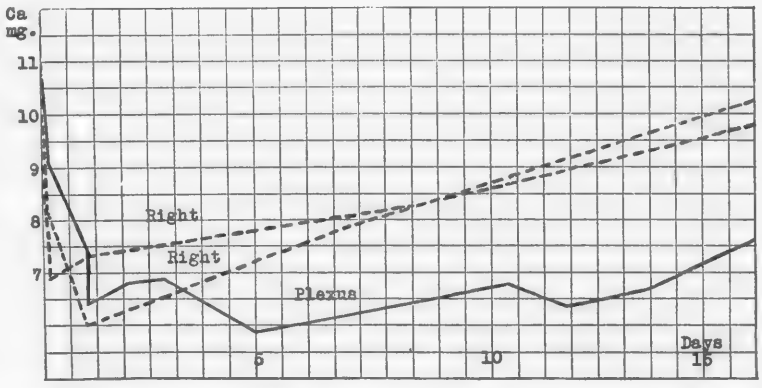

FIG. 1. Calcium of serum following section of the splanchnic nerves or plexus.

nerve. It should be added that the fall in calcium level cannot be attributed to the opening of the abdominal cavity or the handling of the viscera, as was proved by analyses of the blood of animals in which operations were performed to produce chronic intestinal obstruction and bile fistulæ. Following abdominal operations on man it was found that there may be a slight fall in the calcium of the serum, a decrease of less than 1 mg. per cent; only a few estimations of this kind were made. It is perhaps worthy of note that recently Lebedenko (7) has described marked congestion and hemorrhages in the solar plexus following abdominal operations. It will be noted that the summary of operations reproduced in Table IV includes one in which the supra-

renal glands were decapsulated. This operative procedure was followed by a slight decrease in calcium 2 hours later, but nothing further was noted.

In contradistinction to the effect on calcium, the percentage of inorganic phosphorus in the blood, determined by the Bell-Doisy method, was found slightly changed, following the various lesions of the sympathetic nerves.

TABLE IV.

Summary of Operations on Sympathetic Nerves.

Dog		Area of sympathetic nerves severed	Serum Ca		Remarks
No.	Sex		Lowest level	Interval	
			mg. per cent		
8	♀	Few fibers of celiac plexus	8.2	1 day	
9	♂	Left splanchnic	7.5	2½ hrs.	
6	♀	Right "	6.0	25 "	
7	♂	" "	6.9	2½ "	
2	♀	Celiac plexus partially destroyed. Splanchnic cut (?)	8.4	3 "	Hemorrhage, followed by gangrene of intestines
4	♀	Celiac plexus partially destroyed	7.2	1 day	
3	♀	Celiac plexus partially destroyed	5.9	5 days	Low calcium over a 15 day period. No tetany
10	♂	Celiac plexus partially destroyed	6.7	1 day	
1	♀	Cervical sympathetic followed by right splanchnic and part of celiac plexus	6.1	3 days	
5	♂	Suprarenal glands decapsulated	9.6	2 hrs.	

Experiments on the Abdominal Vagi Nerves.

A series of experiments was next carried out to ascertain the effect of section of the vagi nerves on the calcium and phosphorus level of the blood. Under general anesthesia, through an upper median abdominal incision, both nerves were exposed as far cephalad as possible, just as they penetrated the diaphragm. In order to section the

nerves before the abdominal branches were given off traction downward was exerted. A segment of nerve 2 to 3 cm. in length was excised. This procedure had to be carried out with care in order to avoid pneumothorax.

There have been several investigations of the effect of vagotomy on the chemistry of the blood, but these studies have not considered calcium or phosphorus. Cokkalis (8) described a disturbance in the gas exchange of the lungs, an increase of 5 to 7 volumes per cent of the total CO_2 of the arterial blood. Papilian and

TABLE V.

Calcium and Inorganic Phosphate of the Blood Following Bilateral Abdominal Vagotomy.

Dog 11	Date	Interval after operation	Blood			Remarks
			Inorganic P	Serum Ca	Hemoglobin	
	1927		*mg.*	*mg.*	*per cent*	
Mongrel	6/14		3.2	11.3	85	Esophagus pulled down through
♂		1½ hrs.	8.4	11.0	87	diaphragm and both vagi cut;
	6/15	18 "	4.2	10.7		3 cm. excised
8.1 kg.		23 "	3.6	11.0	88	Diet: bread and cooked meat,
	6/17	72 "	3.8	11.3		no milk
	6/21	7 days	4.3	11.1	90	
	6/24	10 "	4.4	11.2		
	6/27	13 "	4.1	12.2		
	7/5	21 "	4.7	10.3		
	7/11	27 "	3.5	10.5		
	7/18	34 "	4.8	10.0		
	7/26	42 "	4.8	11.9		
	9/20	98 "	7.1	11.0		
	9/28	106 "	5.3	9.3		Blood sugar 58.9 mg.
	10/6	114 "	5.18	10.1		" " 93.8 "

Jianu (9) stimulated the sympathetic vagi nerves with suprarenin after section and found that the sugar was increased in both the arterial and the venous blood. Recently Frommel (10) severed the vagi nerves of rabbits and then tested the effect of calcium chloride on the heart; he could find no alteration.

Tables V, VI, and VII present in tabular form the results of three vagotomy experiments. The first shows a rise of calcium to 12.2 mg. 13 days after operation, the second a rise to 14.9 mg. after 8 days, and the third (Table VII) a rise to 13.6 mg. All of these figures are defi-

nitely above the normal. In one instance there was a temporary fall of calcium about 24 hours after the vagi were cut (Table VI). It would seem that the conclusion may be drawn that this operation, in

TABLE VI.

Calcium and Inorganic Phosphate of the Blood Following Bilateral Abdominal Vagotomy.

Dog 12	Date	Interval after operation	Blood			Remarks
			Inorganic P	Serum Ca	Hemoglobin	
	1927		*mg.*	*mg.*	*per cent*	
♂	6/21		4.3	10.3	94	Previous to operation
13.2		1½ hrs.	4.9	10.8	91	Rapid, labored respiration for 15–20
kg.	6/22	19 "	3.9	11.1	90	minutes after recovery from anes-
		24 "	4.2	7.7	90	thesia
	6/23	48 "	4.0	9.1		
	6/24	72 "	3.7	10.7		Diet: bread and cooked meat
	6/27	8 days	4.4	**14.9**		
	7/5	16 "	5.35	11.2		
	7/11	22 "	5.23	10.85		
	7/18	29 "	4.45	10.8		
	9/20	93 "	4.89	10.4		
	9/28	101 "	4.90	10.57		Blood sugar 97.2 mg.
	10/6	109 "	5.32	11.0		" " 71.3 "

TABLE VII.

Calcium, Inorganic Phosphate, and Chloride of the Blood Following Bilateral Abdominal Vagotomy.

Dog 13	Date	Interval after operation	Blood				Remarks
			Inorganic P	Ca	Chlorides	Hemoglobin	
	1927		*mg.*	*mg.*	*mg.*	*per cent*	
♀	6/22		4.0	11.5	360	87	Diet of bread and cooked
Mongrel		1¼ hrs.	2.5	11.4	356	85	meat
11 kg.	6/23	19 "	2.9	10.8	361		
	6/24	48 "	2.5	12.0			
	6/27	5 days	3.8	**13.6**	350		

contradistinction to severing of the splanchnic nerves, leads to an augmentation of the calcium of the blood. It is to be noted also that the equilibrium seems to have been definitely disturbed; we miss the con-

stancy in the calcium level which is such a characteristic feature in almost all normal mammals. In regard to the effect on inorganic phosphate the results are too irregular to warrant a definite conclusion. Attention, however, should be drawn to the sudden and temporary rise of phosphorus in one operation (Table V) from 3.2 to 8.4 mg. 1½ hours after cutting the vagi. In general, it may be stated that this operation tended to bring about a slight disturbance in equilibrium of this ion.

DISCUSSION.

At the present time the physiologic and anatomic conceptions of the sympathetic nervous system are not identical. Whereas physiology has taken account but little of the afferent sympathetic fibers, anatomy has long since ascribed sensory fibers to the heart, mesentery, urinary bladder, etc. Moreover, until recently the centers in the spinal cord and midbrain were not regarded physiologically as a part of the sympathetic system. Nowadays, not only have these areas of the central nervous system been included, but the concept has been broadened functionally, so as to include some of the glands of internal secretion. In the present state of our knowledge it would be unwise to attempt to interpret the pathogenesis of the chemical changes which we have brought about by a destruction of these nerve paths. It would be of interest to know what has become of the calcium which is lacking in the blood in such marked degree. Has this deficiency come about from a failure of absorption, from an increased excretion by way of the bowel or the urinary tract, or does this loss represent merely an altered distribution in the tissues of the body? This question is of significance and we hope that further investigation will be able to shed light upon it. It cannot be ascribed to a mere alteration in the concentration of the blood for, as may be noted, percentages of hemoglobin before and after the various operations were but little altered. Whatever may be the physiologic mechanism involved, it would seem that in connection with the development of metabolic disturbances the rôle of the nervous system may well be of greater importance than is generally believed. In tetany, for example, we cannot take it for granted that nerve hyperirritability results solely from deficiency—either absolute or relative—of calcium, but must investigate whether the disturbance of nerve function may also be a primary factor.

CONCLUSIONS.

When a splanchnic nerve or the celiac plexus was severed a definite fall in serum calcium resulted, the level falling to 6 mg. per cent and remaining low for 1 or 2 weeks. Tetany, however, did not develop. The inorganic phosphate was slightly altered.

On the other hand, when the vagi nerves were severed, the serum calcium rose.

The equilibrium of both calcium and phosphorus was rendered less stable by the division of the sympathetic or the parasympathetic nerves.

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ALTERATIONS IN THE CALCIUM LEVEL OF THE BLOOD FOLLOWING SECTION OF THE SPINAL CORD.*

By ALFRED F. HESS, M.D., BENJAMIN N. BERG, M.D., AND ELIZABETH SHERMAN.

(*From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York.*)

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In connection with an investigation of the effect of nerve injury on the calcium and phosphorus content of the blood, it seemed of advantage to ascertain the effect of section of the spinal cord at various levels. As far as we know, a study of this kind has not been carried out.

It will be remembered that about 50 years ago Charcot aroused the medical world by a clinical description of lesions of the bones resulting from injury to the spinal cord—from pressure of carious vertebræ, tumors, etc., or associated with organic disease of the nervous system. Not only the bones but the tendons, ligaments, and the surrounding connective tissue were involved frequently in these pathologic changes. Since this time a variety of similar alterations have been described by clinicians and pathologists. . In addition to lesions of the long bones, stone in the kidney has been noted from time to time in conjunction with fracture or injury of the spinal vertebræ. In 1895 Mueller (2) reported 10 cases of fracture of the spine, associated with prolonged paralysis, in which calculi were found in the kidneys upon postmortem examination; in many of these instances the compression was mild. Mueller believed that the calculi had developed as the result of a lesion of the nerves. However, as renal and bladder disturbances, as well as infection frequently accompany injuries to the spinal cord, it is difficult to judge whether these factors did not play the predominant rôle in the formation of the calculi. Some pertinent observations made in the course of the World War should be mentioned in this connection. It was found by army surgeons that wounds involving the spinal cord, resulting in paraplegia, were followed often by calcification of the joints and the surrounding tissues. By far the most complete report treating of this condition is that of the French investigator Ceillier (3) who published a monograph entitled "Para-osteo-arthropathies" in which he detailed 79 cases of

* Presented at the Annual Meeting of the American Society of Biological Chemists, Rochester, April 14, 1927 (1).

this kind, occurring among 160 paraplegics. The cases which resulted from bullet wounds, shrapnel, bombs, falls, etc. were studied by means of radiographs. Calcification resulted apparently irrespective of the level of the cord lesions; the highest injury was at the second dorsal vertebra. In all instances the lesion was severe, leading to complete or almost complete paralysis and loss of sensation in the extremities. Similar, but less convincing observations were published from the records of the British Army. More recently Meyer (4) has reported an interesting case of compression fracture at the eleventh dorsal vertebra with contusion of the upper lumbar cord. Six months later symmetrical formation of bone developed in the connective tissue of both quadriceps muscles and the adjacent fascia and periosteal tissue, and 10 months after the injury large calculi were found in both kidneys.

EXPERIMENTS.

Dogs were placed under complete ether narcosis and a laminectomy was carried out in the typical way, and the cord transected at the desired level. The technique was not complicated and the animals withstood the operations well. Following transection, the rent into the dura was sutured in order to avoid leakage of spinal fluid. The level at which the incision was made was checked at postmortem examination and at times found to have been slightly higher or lower than had been anticipated. The bleeding was moderate. A flaccid paralysis of both hind legs came about rapidly and was associated at times with a transient spasticity of the fore legs. The respiration and swallowing movements remained unaltered and nourishment was well taken. Precautions were taken against chilling. The animals were bled from the jugular vein before operation and as a rule 2 hours subsequently and at varying intervals during the next few days.

The results of typical successful operations are given in Tables I and II. The first shows that the percentage of calcium in the serum was 9.6 mg. previous to operation, rising to 17 mg. 22 hours later, and was 16.4 mg. 46 hours subsequent to the operation. The Kramer-Tisdall method was employed for estimations of calcium and the Briggs modification of the Bell-Doisy method for inorganic phosphorus. At necropsy the cord was found completely severed between the first and second dorsal segments except for a few fibers anteriorly. A similar result was obtained in the operation, of which the details are summarized in Table II. In this instance the calcium content rose to 13.2 mg. 2 hours and 15 mg. 21 hours subsequent to division of the cord. It will be noted that the equilibrium of the calcium was also markedly disturbed, that for some days it fell even below the normal level but that gradually it regained

the normal percentage of between 9 to 10 mg. It must be added, however, that these striking chemical changes could not be brought about at will. In spite of the fact that section of the cord was made in three other animals apparently at the same plane, little or no fall in the percentage of calcium resulted. The probable explanation for this irregularity is that although the operative procedure was seemingly identical, the lesion of the nerve cells was essentially different. Experiences of this kind are not uncommon in the course of experimental surgery on the spinal cord and brain.

TABLE I.

Calcium and Inorganic Phosphate of the Blood Following Transection of Spinal Cord between First and Second Dorsal Segments.

Dog 1	Date	Interval after operation	Blood		Remarks
			Inorganic P	Serum Ca	
	1927	*hrs.*	*mg.*	*mg.*	
Mongrel	2/10		3.62	9.6	Previous to operation
♂		2	2.98	9.6	Good recovery. Complete flaccid paralysis below level of lesion
9.2 kg.	2/11	22	3.35	17.0	Milk feeding
	2/11	27	3.40	10.0	
	2/12	46		16.41	
	2/14				Died. Autopsy showed infection of wound; cerebrospinal fluid clear. A few anterior fibers of cord not severed. Parathyroid glands normal macroscopically and microscopically

Noting that Ceillier attributed the calcification in his cases to an injury of the sympathetic nerves supplying the blood vessels and to a consequent edema and hyperemia, we made an attempt to reproduce the effect of the bullet and shrapnel wounds. With this in view the cord was traumatized at different levels below or in the neighborhood of the incision. This procedure, which was carried out with the animals under complete anesthesia, was without effect.

In other instances, the spinal fluid was allowed to escape freely from the canal and in still others the nerve roots were resected in addition to cutting the spinal cord. For example, in one operation where the

cord was divided between the first and second dorsal segments, the nerve roots of the first, second, and third dorsal areas were divided likewise on both sides. A similar procedure was carried out in connection with the division of the cord between the first and second lumbar segments, as well as at the eighth cervical segment. It seems unnecessary to record the percentages of calcium in these various

TABLE II.

Calcium and Inorganic Phosphate of the Blood Following Transection of Spinal Cord between First and Second Dorsal Segments.

Dog 2	Date	Interval after operation	Blood		Remarks
			Inorganic P	Serum Ca	
	1927	*hrs.*	*mg.*	*mg.*	
♂	2/15		3.46	9.9	Previous to operation
8.8 kg.	2/15	2	3.60	13.2	Good recovery. Complete flaccid paralysis below level of lesion. Incontinence of urine
	2/16	21	3.44	15.0	Milk 100 cc.
		26		11.1	
	2/17	44	4.02	6.1	Wound infected. Milk 500 cc.
		52	3.86	10.1	
	2/18	74	3.94	7.9	Condition good. Pulse 125, respirations 14. Milk 600 cc., bread 10 to 20 gm.
		days			
	2/19	4		11.0	Blood CO_2 49.95. Condition good. Pulse 116, respirations 60, temperature 38°. Reflexes hyperactive. Milk 400 cc.
	2/20	5		9.6	Pulse 120, respirations 70, temperature 38.2°. Milk 400 cc.
	2/21	6	3.78	9.8	Temperature 38°, respirations 22. Milk 250 cc.
	2/22	7		9.0	Killed. Wound infected. No meningitis. No pneumonia

operations. The results can be summarized by the statement that there was no significant change.

In two instances the cord was divided in two places. In one animal section was made first at the upper dorsal segment and some days later at the seventh cervical level, and in another it was cut first between the seventh and eighth cervical segments and 2 days later at the

third dorsal level. In neither case was the calcium level altered significantly. In fact, among a total of fourteen operations on the cord at various levels, definite and striking changes could be brought about only following section at the upper dorsal segments.

It was thought that the disturbance in the calcium might be due to an effect on the parathyroid glands through the nerves which regulate their activity. The nerve supply of the parathyroids has not been worked out with the same completeness as that of the thyroid gland. In order to test this hypothesis the thyroid and parathyroid glands were removed to observe whether the diminution of calcium which is

TABLE III.

Thyroparathyroidectomy Followed by Transection of Spinal Cord at Third Dorsal Segment.

Dog 3	Date	Interval after operation	Blood		Remarks
			Inorganic P	Serum Ca	
	1927	*hrs.*	*mg.*	*mg.*	
Mongrel	4/19		2.86	10.1	*Thyroidparathyroidectomy*
♀		2½		8.2	
12 kg.		5		7.8	Pulse 80, respirations 60, temperature 38.5°
	4/20	22½	2.74	8.0	Pulse 110, respirations 80, temperature 38.5°. No tetany. Sensitive to noises
	4/20	24			*Section of spinal cord*
		1	3.52	11.1	Hoarse barking. Respirations 35 to 72
		5	3.51	6.7	Rapid shallow breathing. 400 cc. water
	4/21	21		6.2	500 cc. water. Killed

brought about by this means could be raised by subsequent division of the spinal cord at the upper dorsal segment. Table III shows the result of this procedure. It will be noted that 5 hours subsequent to parathyroidectomy the serum calcium had fallen from 10.1 to 7.8 mg. per cent, and a condition of latent tetany had developed. The following day the cord was sectioned at the third dorsal segment. One hour later the calcium had risen to somewhat above the original percentage, namely to 11.1 mg. In other words, the rise in calcium which followed division of the spinal cord was not to be ascribed to a stimulative effect on the parathyroid glands and appeared quite independent of them.

It is impossible at present to explain the marked alteration in the percentage of calcium resulting from section of the spinal cord. The only procedure which is known to produce a similar effect is the injection of parathyroid extract. It would seem as if the alteration must be due to a disturbance of the sympathetic system. The anatomy of these nerves and centers within the spinal cord has not, however, been determined with sufficient accuracy to enable one to estimate the validity of this interpretation Some years ago Biedl (5), in a paper on the splanchnic centers, described spinal centers of the sympathetic at the boundary between the cervical and dorsal cord, concentrated mainly between the eighth cervical and second dorsal but extending down even to the fifth dorsal segment. These cells varied in their sites within the cord at different levels. More recent anatomic studies of the spinal centers of the vegetative nervous system have served to locate them in the intermediary lateral tract of the cord. According to Mueller (6), who recently has reviewed this subject, no studies of these cells have been carried out in cases of tabes or of syringomyelia. It would seem that at the present time little can be gained by bringing forward further hypotheses in connection with the phenomenon which we have described.

CONCLUSIONS.

A marked rise and disturbance in equilibrium of the calcium in the blood was brought about by section of the spinal cord in the upper dorsal segment. This reaction, however, was not constant.

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RECENT STUDIES ON METHODS OF ISOLATING A BACTERIOPHAGE FOR BACILLUS DIPHTHERIÆ.

By JULIUS A. KLOSTERMAN and KATHRYN W. SMALL, M.D.

(*From the Laboratories of the Department of Bacteriology, College of Dentistry, New York University, New York.*)

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D'Hérelle, Fejgin and Blair (1–3) have published accounts of their success in isolating a lytic principle for *B. diphtheriæ*. In the light of increasing interest in studies of bacteriophagy in general, it appears advisable to contribute to the available literature additional experimental data pertaining to the isolation and activity of a true bacteriophage for *B. diphtheriæ*.

The writers have exercised particular care to utilize only toxic strains of *B. diphtheriæ* throughout their entire investigation. The purpose was to secure a phage active against virulent strains, to be used in proposed subsequent experiments.

Unfortunately, d'Hérelle has given insufficient information concerning the details of his investigation on antidiphtheria bacteriophages. Hence duplication of his researches for corroboration could not be attempted. However, the two phages which he reported to have isolated from the feces of diphtheria antitoxin horses were active only upon atoxic strains of *B. diphtheriæ*. The mode of detecting the bacteriophagic activity was not disclosed in the instance of this particular phage.

Fejgin briefly describes having isolated a diphtheria phage from a Martin bouillon culture of *B. diphtheriæ* several weeks old. In an effort to demonstrate the activity of this phage on agar, she secured but a single plaque, in the center of which one bacterial colony subsequently developed. Unfortunately she was unable to proceed with her investigation as the tube was broken in handling.

Blair, on the other hand, reported isolations of antidiphtheria phages with comparative ease from three distinct sources. He based these findings entirely upon the degree of clearness of broth cultures containing the filtrate to be tested for phage, as compared to the turbidity of the broth culture controls. In no instance

has he included accounts of antidiphtheria phage activity on agar. The latter characteristic, according to the most recently developed technical procedure, appears to be absolutely necessary in establishing the fact of true bacteriophagy. Lytic action based wholly upon apparent lysis in broth cultures may be due, particularly if not capable of serial transfer, to inhibitory or enzymatic substances which by chance are present in the suspected filtrates rather than to a real bacteriophage. Another extremely important factor which also decides the genuineness of a bacteriophage is the well known characteristic of cultivation by successive transferring with the microorganism for which it is active. Blair gave no indication that his phages were propagatable.

The following protocols outline the procedure used in the attempt to isolate an antidiphtheria phage. Four cardinal principles were rigidly adhered to—first, the use of only definitely toxic strains, second, requiring all suspected phages to show activity on agar slants as well as in broth, third, carrying all tests on filtrates through two or more transfers before discarding because of lack of visible phage action, fourth, requiring that any suspected phage be capable of propogation by subculturing in the presence of a toxic strain of *B. diphtheriæ*. Fecal material collected from either human or animal sources was treated as follows. Approximately a cc. of feces was placed in 20 cc. of broth (pH 8.2) and allowed to incubate overnight at 37°C. It was then filtered through infusorial earth packed in a Buchner funnel. The filtrate was finally passed through a Berkefeld filter (*w*).

The first attempt at isolation was made by using the feces of an active case of diphtheria.[1] The infecting organism was isolated from the throat of this patient and was made use of in the following procedure. It is referred to as the homologous strain. Eleven stools were examined. The first specimen was taken on the 5th day of illness and the others at intervals for the next 2 weeks covering course and convalescence. Of the eleven stool filtrates eight were tested upon one homologous and two heterologous strains. Two were tested upon one homologous and five heterologous strains. One was tested upon one homologous and six heterologous strains. In no instance was there any indication of the presence of a phage.

Next a 33 day old culture in 30 per cent serum broth of a recently isolated virulent strain of *B. diphtheriæ* (Strain *A*) was filtered and used

[1] The authors wish to thank Dr. W. H. Park for his kindness in making arrangements to secure the materials used in these experiments.

as a potential source of a phage. It was tested against the seven strains of *B. diphtheriæ* which included its homologous strain and gave entirely negative results.

The third source tested for the presence of an antidiphtheria phage was the intestinal contents and peritoneal washings of guinea pigs inoculated with diphtheria cultures (Tables I and II).

TABLE I.

Test Animals.

Showing animal inoculations, time of death, result at autopsy with respect to signs of diphtheria toxemia and result of test for phage activity on intestinal contents and peritoneal washings.

Infecting strain used	Broth suspension, 1 cc.										Broth culture, 1 cc.				
	Intraperitoneal					Subcutaneous					Subcutaneous				
	Guinea pig No.	Death	Autopsy findings	Phage activity: Intestinal contents	Phage activity: Peritoneal washings	Guinea pig No.	Death	Autopsy findings	Phage activity: Intestinal contents	Phage activity: Peritoneal washings	Guinea pig No.	Death	Autopsy findings	Phage activity: Intestinal contents	Phage activity: Peritoneal washings
		hrs.					*hrs.*					*hrs.*			
B. diphtheriæ Strain *M* 1314	1	48	+	−	−	4	48	+	−	−	6	48	+	−	−
B. diphtheriæ Strain *A*	2	24	+	−	−	5	48	+	−	−	7	Killed at 48	−	−	−
B. diphtheriæ Strain *F*	3	48	+ (also peritoneal abscess)	−	−						8	48	+	−	−

Eleven animals were used of which eight served as test pigs and three as controls. Three toxic strains of *B. diphtheriæ* were employed, namely Strain *M* 1314, Strain *A* and Strain *F*. The first two had been isolated 7 months ago while the third was isolated 3 weeks previously. Three different types of inoculations were made in the case of two strains, while only two of these were employed with the third strain. Hence altogether eight guinea pigs were used as test animals. The types of inoculations resorted to were first, 1 cc. of a suspension made by emulsifying the bacterial mass of an 18 hour serum agar slant in 5 cc. of broth, adminis-

tered intraperitoneally; second, the same material administered subcutaneously and third, 1 cc. of a 48 hour broth culture given subcutaneously.

One control pig was used for each of the three strains of *B. diphtheriæ*. In each case the pig received a subcutaneous inoculation of 1 cc. of a 48 hour broth culture of its assigned strain and in addition two hundred units of diphtheria antitoxin given intraperitoneally. These controls served as an indirect check on the toxin-producing property of the listed strains. In other words they were included for the purpose of demonstrating that all of the strains used produced toxin which was neutralized by a specific antitoxin.

The results were as follows. One test animal (No. 2) receiving an intraperitoneal inoculation of a suspension of Strain *A* died in 24 hours. The autopsy revealed signs of diphtheria toxemia which were the presence of a serous exudate in the peritoneal cavity, congestion of the peritoneum and enlargement and congestion of the adrenals. Six other test pigs (Nos. 1, 3, 4, 5, 6, 8) died in 48 hours with

TABLE II.

Control Animals.

Showing animal inoculations used to control specificity of toxin produced by listed strains.

Infecting strain used	Diphtheria antitoxin administered subcutaneously	Guinea pig No.	Broth culture (subcutaneous)	
			Death	Autopsy findings
B. diphtheriæ Strain *M* 1314	200 units	9	11 days	Cause of death undetermined (no signs of diphtheria toxemia)
B. diphtheriæ Strain *A*	200 units	10	4 days	Streptococcus infection (no signs of diphtheria toxemia)
B. diphtheriæ Strain *F*	200 units	11	Alive	

positive signs at autopsy. The remaining test pig (No. 7) having received a subcutaneous broth inoculation of Strain *A* was killed and autopsied with negative results, however.

One control (No. 11) was alive on the 20th day. A second control (No. 10) died on the 4th day. Before inoculation the latter pig showed a fluctuating swelling of the perineal region. At autopsy there were no signs of diphtheria toxemia. However, there were soft swollen inguinal and retroperitoneal lymph nodes (bilateral) and an enlarged, congested spleen. Smears from the lymph nodes showed many Gram-positive cocci in long chains. Hence the animal probably succumbed to an acute streptococcus infection, the focus of which was the lesion observed before inoculation. The third control (No. 9) died on the 11th day and autopsy failed to reveal the cause of death but certainly there was no indication that it was due to diphtheria toxemia.

At autopsy one of the intraperitoneal suspension pigs (No. 3) showed a small peritoneal abscess at the site of inoculation. A smear and culture from the lesion showed *B. diphtheriæ*. The abscess and surrounding wall were emulsified in broth, incubated and filtered.

This filtrate and those made from intestinal contents and from peritoneal washings of the eight pigs which received culture without antitoxin were examined

TABLE III.

Results of phage activity of filtrates of feces from antitoxin horses on strains of *B. diphtheriæ*.

Results indicated are those recorded after two transfers.

Strains of *B. diphtheriæ*	Filtrates from feces of antitoxin horses					
	149	152	161	87	143	144
B	—	—	—	—	—	—
C				—	—	—
D				—	—	—
E				—	—	—
Park 8	—	—	—	—	—	—
M 1314	—	—	—	—	—	—

Blank space indicates these combinations were not tested.

TABLE IV.

Results of phage activity of filtrates of feces of five additional antitoxin horses on seven strains of *B. diphtheriæ*.

Results indicated are those recorded after two transfers.

Strains of *B. diphtheriæ*	Filtrates from feces of antitoxin horses				
	165	142	156	136	154
A	—	—	—	—	—
B	—	—	—	—	—
C	—	—	—	—	—
D	—	—	—	—	—
E	—	—	—	—	—
Park 8	—	—	—	—	—
M 1314	—	—	+	—	—

for the presence of phage. Each filtrate was tested on its homologous strain and gave negative results. Then all filtrates were tested on two heterologous strains of *B. diphtheriæ* and six of the filtrates were additionally tried on six other heterologous strains of *B. diphtheriæ*. All results were negative (Table I).

The final attempt to isolate an antidiphtheria phage was conducted by using the filtrates of feces emulsions of antitoxin horses. The feces of six horses[2] retained at Otisville (for the New York City Department of Health) for the production of diphtheria antitoxin were emulsified, incubated and filtered according to the usual procedure. Of these six filtrates three were tested on three strains of diphtheria and three were tried on six strains. As the toxin of Strain Park 8 is used for the stimulation of antitoxin in these horses, it is regarded as the homologous strain. All efforts to isolate a phage from these filtrates failed (Table III).

TABLE V.

Observations on specificity of two generations of antidiphtheria Phage 156 *M* 1314 on Strains of *B. diphtheriæ*.

Results indicated are those recorded after two transfers.

Strains of *B. diphtheriæ*	Antidiphtheria Phage 156 *M* 1314 Generation 11	Antidiphtheria Phage 156 *M* 1314 Generation 23
A	−	−
B	−	−
C	−	−
D	−	−
E	−	−
F		+
G		+
Park 8	−	−
M 1314	+	+

Blank space indicates these combinations were not tested.

Filtrates from five other horses immunized with diphtheria toxin showed activity against one of these strains. This is the only instance in which the authors secured an antidiphtheria phage (Table IV).

The filtrate of Horse 156 showed lysis of *M* 1314 in broth and on agar in the second generation. The test broth was only slightly less turbid than the control and on agar phage activity was evident only in the form of isolated plaques. The fourth generation showed confluent plaques on the agar. The successive generations in broth gradually showed more and more lysis but even in the twenty-second

[2] The toxin used to immunize these horses consisted of the supernatant fluid of broth cultures which were allowed to sediment.

generation lysis is not complete. 500 units of concentrated diphtheria antitoxin were mixed with culture and phage in broth to counteract any undesirable action of toxin upon phage activity. There was

TABLE VI.

Observations on specificity of antidiphtheria Phage 156 *M* 1314 tested on eighteen strains of microorganisms other than toxic *B. diphtheriæ*.

Results indicated are those recorded after two transfers.

Microorganisms	Antidiphtheria Phage 156 *M* 1314 (Generation 16)
Diphtheria-like Strain 1154 (non-toxic)	—
Diphtheria-like Strain 1175 (non-toxic)	—
Diphtheria-like Strain 1178 (non-toxic)	—
Diphtheria-like Strain *U B* (non-toxic)	—
B. xerosis	—
B. hoffmanii	—
Staphylococcus aureus	—
Staphylococcus albus	—
Staphylococcus citreus	—
B. coli communior	—
B. dysenteriæ Shiga	—
B. dysenteriæ Flexner	—
B. dysenteriæ Mt. Desert	—
B. typhosus (Pfeiffer)	—
B. paratyphosus A	—
B. paratyphosus B	—
Cholera-like	—
B. subtilis	—

TABLE VII.

Observations on thermal inactivation point of antidiphtheria Phage 156 *M* 1314.

Temperature	30 min. exposure	45 min. exposure
°C.		
50	Active	Active
55	—	—
60	—	—

no apparent difference in end-result between the test containing antitoxin and the control without antitoxin as far as lysis due to phage activity was concerned.

Two later specimens of feces from Horse 156 were tested for the presence of phage and were found negative.

The blood serum of Horse 156 whose first specimen of feces gave positive results on *M* 1314 was tested on this culture but failed to give evidence of a phage.

The specificity of Phage 156 *M* 1314 was studied. The eleventh generation was tested on six diphtheria strains other than Strain *M*

TABLE VIII.

Results of using non-diphtheria phages in conjunction with *B. diphtheriæ* Strain *M* 1314 for determining whether or not these known bacteriophages are active on this strain.

Results indicated are those recorded after two transfers.

Name of phage	Source of phage	Organism on which phage is active	Generation of phage	Activity on *B. diphtheriæ* Strain *M* 1314
Coli	Not recorded*	*B. dysenteriæ* Mt. Desert	10	–
Coli	East River water	*B. coli communior*	5	–
Coli	Bellevue sewer water	*B. coli communior*	5	–
Coli	Willard Parker sewer water	*B. coli communior*	7	–
Coli	Normal cat feces	*B. coli communior*	8	–
Dysentery	Feces from case of typhoid	*B. dysenteriæ* Shiga	5	–
Dysentery	Sample from pail of milk just collected by farm hand	*B. dysenteriæ* Shiga	9	–
Dysentery	Human saliva pooled from six sources	*B. dysenteriæ* Shiga	12	–
Typhoid	Feces from case of dysentery	*B. typhosus* (Pfeiffer)	6	–

* This phage was supplied through the courtesy of Dr. J. J. Bronfenbrenner to whom the authors are also indebted for valuable suggestions and for his kindness in reviewing this paper.

1314, with negative results. The twenty-third generation was tested on the same six strains and two additional ones. It was active against the latter two. These two strains, namely Strain *F* and Strain *G* as well as Strain *M* 1314, were all recently isolated; that is within 6 months (Table V).

The sixteenth generation of antidiphtheria Phage 156 *M* 1314 was tested on eighteen different strains of microorganisms other than toxic *B. diphtheriæ* with negative results (Table VI).

The thermal inactivation point of Generation 23 of antidiphtheria Phage 156 *M* 1314 was found to lie between 50°C. and 55°C. (Table VII).

Generation 22 was titrated by a modified broth dilution method (4). In carrying out the final transfer, however, the authors substituted filtration for heating as this phage is inactivated at a comparatively low temperature. The phage showed activity when it was present in a 1×10^{-9} dilution.

Nine non-diphtheria bacteriophages of high titer previously isolated from various sources were tested for the purpose of determining whether or not they were active on *B. diphtheriæ* Strain *M* 1314 (Table VIII).

SUMMARY.

Of the attempts to isolate an antidiphtheria phage (1) from stools collected daily during the course of a case of the disease, (2) from a 33 day old broth culture of *B. diphtheriæ*, (3) from intestinal contents and peritoneal washings of guinea pigs inoculated with three different toxic strains of *B. diphtheriæ*, none yielded an antidiphtheria phage. However of eleven specimens of feces collected from eleven antitoxin horses one was found to contain a bacteriophage active against *B. diphtheriæ*. This phage was not observed in the first generation and did not show up until transferred the second time. Had the results of the first transfer been regarded as final in all certainty the existence of a phage would not have been recognized. Two additional specimens of feces from the positive horse (No. 156) were later tested to determine whether this bacteriophage was continually present in the intestinal tract of this animal. Both of these feces filtrates failed to yield a phage. Later a sample of freshly collected blood serum from the same horse was tested and found not to contain a phage.

The antidiphtheria phage was tested against eighteen non-diphtheria strains of microorganisms to determine whether it would show lytic activity for related or unrelated bacteria. There was no evidence of lysis in any of these types. The specificity of this phage was also tested on nine strains of *B. diphtheriæ* and found to be active on three heterologous strains (Strain *M* 1314, Strain *F* and Strain *G*). These incidentally were all recently isolated. It failed to lyse the remaining six strains of which five were recently isolated.

B. diphtheriæ Strain *M* 1314 was used in combination with nine heterologous bacteriophages isolated from various sources, to determine if any of these phages would by chance lyse this culture. The results were all negative.

To date this phage fails to show complete lysis although the twenty-second generation has a titer of 1×10^{-9}.

Additional proposed experiments involving this phage are being planned and will be undertaken in the near future.

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THE INFLUENCE OF HEATING THE IMMUNIZATION MATERIAL UPON THE ANTIBODY-INVOKING EFFECTIVENESS OF THE TYPE-SPECIFIC AND SPECIES-SPECIFIC ANTIGENS OF TYPE II PNEUMOCOCCUS CELLS.

By EMIDIO L. GASPARI,* JOHN Y. SUGG, WILLIAM L. FLEMING, and JAMES M. NEILL, Ph.D.

(*From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.*)

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INTRODUCTION.

Avery and Heidelberger and their associates (1–4) have presented convincing evidence that the virulent pneumococcus cell contains two different types of antigens: (1) a type-specific antigen (SP) which gives rise to a type-specific antibody; (2) a species-specific antigen (P)[1] which gives rise to a species-specific antibody. The type-specific antigenic complex is contained in the intact cell of strains possessing the soluble specific substance; immunization of a suitable animal with suspensions of pneumococcal cells invokes a type-specific antibody which reacts specifically with the soluble specific substance whether contained in solution (S-precipitation) or disposed on the periphery of the bacterial cell (agglutination); the same antibody is also apparently the one responsible for the passive protection of mice, at least against Type II pneumococci (5). The species-specific antigen, in contrast to the type-specific one, gives rise to an antibody

* Mr. Gaspari's cooperation in this work was made possible by a grant from The Henry Strong Denison Medical Foundation.

[1] The term species-specific "antigen" is used in the inclusive sense of Avery and Heidelberger (1, 2) to refer to the protein (mainly nucleoprotein and mucoid) which reacts with the species-specific precipitins that are invoked by immunization with protein derived from all types of pneumococci. It is not important in this paper that the protein may include a number of separate but unrecognized antigens, provided they are sufficiently alike in chemical and physical properties to be affected in similar manner by heat.

reactive with the protein derived from all types of pneumococci. This antibody is especially prominent in the immune serum obtained by the injection of solutions of the pneumococcus nucleoprotein (3) or of filtered solutions of the endocellular substances which are liberated when pneumococci are dissolved by bile or disintegrated by freezing and thawing (4). The prominence of these species-specific antiprotein precipitins in the immune serum invoked by solutions of pneumococci is due to the absence of the type-specific antibody which is dominant in the usual antibacterial serum obtained by injection of the intact cells. However, certain amounts of the species-specific antibody are also present although seldom so prominent, in the type-specific immune serum.

Although both anti-S and anti-P occur together in the usual antibacterial serum, sufficient evidence (1–4, 6) has been presented to prove that each of the antibodies is distinct and separate and that each is invoked by a distinct and separate antigen. The present investigation proceeds upon that basis, and consists of a comparison of the effect of heating of the immunization material (suspensions of pneumococci) upon the antibody-invoking property of the type-specific (SP) and species-specific (P) antigens of Type II pneumococcus cells.

Any comparison of the influence of heat upon the antibody-invoking effectiveness of antigens should take into account the size of the doses injected into the animals, the total amount of antigenic material, number of injections or stimuli, and the length of time of the immunization. Although these factors are frequently ignored, it is obvious that comparisons of the effectiveness of unheated antigen with heated antigen are of little value if the dosages of total antigen are greatly in excess of that required for the maximum immunity response of the animal (7). All these factors were carefully controlled in our experiments; the animals were immunized with two different dosages and the sera tested at different stages of the immunization. While individual differences are to be expected, and were encountered, in the degree of responses of the individual rabbits, a sufficient number of rabbits (41) were included to furnish a valid basis for a comparison of the influence of heat upon the effectiveness of the antibody-invoking property of the type-specific and species-specific antigenic constituents of Type II pneumococci.

In one sense, the species-specific antigen (P) can be considered an endocellular antigen contained inside of the type-specific pneumococcal cell. Since these bacteria autolyze readily, the suspensions of pneumococci employed in routine immunizations contain significant amounts of free intracellular substances in solution; and, as pointed out by Avery and Heidelberger (2), animals injected with such material receive, in fact, a mixture of varying proportions of free or dissociated P together with the undissociated type-specific antigenic complex. This free P can be expected to give rise to species-specific antibody and thus the amount of autolysis which has occurred *in vitro* before injection of the material is certainly a factor in determining the species-specific antibody response. Precautions to avoid the presence of autolytic products were especially necessary with the unheated bacteria which autolyze more rapidly *in vitro* than do the heated cells, and for this reason fresh bacterial suspensions were prepared at least every 2nd day. However, in spite of all precautions, it is doubtful whether it is possible to obtain a suspension of pneumococcus cells, either heated or unheated, in which no cellular changes (freeing of the P) have occurred. Since dissolution of pneumococci takes place in the animal body, *in vivo* lysis should also be considered a possible source of the species-specific antigen when animals are immunized with suspensions of pneumococcus cells (2). In the present investigation, both possible sources of the species-specific antigen are considered since both filtered solutions of intracellular substances and vaccines with the minimum of autolysis were employed as immunization material.

EXPERIMETAL.

Tests of the Immune Sera.—The immune sera were tested for type-specific and species-specific antibodies by the same general methods as those employed at the Hospital of The Rockefeller Institute (6). The type-specific antibody (anti-S) was recognized by three methods: (1) precipitation of the soluble substance from solution; (2) type-specific agglutination of broth cultures of the homologous strain; (3) passive protection of mice (8). The precipitation of the soluble substance (S) was tested with filtrates of young, unautolyzed broth cultures of pneumococci; the substitution of these fluids for solutions of the purified S substance being justifiable since they were devoid of the species-specific protein and contained no other detectable serologically reactive substance other than the type-specific S which is liberated in the early growth of pneumococci. The tests for the species-specific antiprotein precipitin were made with a filtered solution of Type I pneumococcus cells in which the species-specific antigen is the only substance reactive with Type II immune serum.

Experiment 1. Influence of Heat upon the Antibody-Invoking Properties of the Protein Contained in Solutions of Pneumococcus Cells.

Solutions of pneumococcus cells lack entirely the effective antigenic complex which is responsible for the production of the type-specific antibody invoked

by suspensions of intact pneumococcus cells and hence the dominant antigen contained in the bacterial solutions is the species-specific protein (P) which gives rise to anti-P precipitins. Although our primary interest was in a study of the conditions determining the relative proportion of the two types of antibodies (anti-S and anti-P) invoked by the intact bacterial cells (vaccine), the first step in the investigation consisted of a study of the influence of heat upon the antibody-invoking property of the protein as contained in the cellular solutions. Previous experiments (9) had shown that the anti-protein precipitin response was slightly diminished by heating pneumococcus solutions for 10 minutes at 55°C. and greatly decreased by boiling. In the following experiment, the bacterial solutions were heated for 30 minutes at 55°, 75°, 100°, and 120°C., and the antibody-invoking activity of the heated solutions was compared with that of unheated pneumococcus solutions. In order to make the comparison more valid, two test doses were employed, one of which was 20 times as great as the other; the use of the two dosages furnishing a basis to compare the antigenic effectiveness of small amounts of the unheated solution with the effectiveness of large doses of the heated material. Test bleedings were made after two and after five courses of injections; the examination of the sera after two different periods of immunization furnishing a basis for comparison of the effectiveness of unheated and heated antigen from the standpoint of the relations of the total amount of antigen and of the number of injections required to invoke comparable immunity responses. The results are summarized in Table I.

The results of this experiment (Table I) showed that heating for 30 minutes at any temperature between 55° and 120°C. reduces the antibody-invoking activity of the species-specific protein contained in solutions of pneumococcus cells. Two courses of injections with a small dose (0.1 cc.) of the unheated solution invoked a more effective response than was obtained by immunization with the heated material, not only when the doses of unheated and heated material were equal but also when the dosage of heated material was 20 times as great as that of the unheated antigen solution. The same effect was evident when the immunization was continued to a total of five courses of injections: although the prolonged immunization with heated solution caused some increase in the anti-P precipitin content of the sera, the antibody responses to the heated antigen were always smaller and approached that invoked by the unheated material only in animals which had received doses 20 times as large as those required when the unheated solution was used. Thus, a comparison of the response to the unheated pneumococcus solution with the response to the heated solutions, shows that 30 minutes exposure to temperatures from 55°

to 120°C. diminished the effectiveness of the species-specific protein antigen, both from the standpoint of the degree of antibody response to equal doses of unheated and of heated solution, and also from the standpoint of the amount of total antigen (dosage) and number of injections required for an effective response.

TABLE I.

Influence of Heat upon the Antibody-Invoking Properties of Solutions of Pneumococcus Cells.

Antigen used in immunization: Pneumococcus cell solution		Species-specific protein precipitins (anti-P)				Type-specific antibodies (anti-S)
		Serum after 2 courses of injection		Serum after 5 courses of injection		Serum after 5 courses of injections
Treatment of antigen	Size of dose injected	Protein solution				Agglutinins Anti-S precipitins Passive protection
		Undiluted	1/10 diluted	Undiluted	1/10 diluted	
	cc.					
Unheated	0.1	++	+	+++	++	0
Heated 30 min. at 55°C.	0.1	±	0	+	±	0
	2.0	+	±	++	+	0
Heated 30 min. at 75°C.	0.1	0	0	±	0	0
	2.0	+	+	++	+	0
Heated 30 min. at 100°C.	0.1	0	0	±	0	0
	2.0	+	±	+	±	0
Heated 30 min. at 120°C.	0.1	0	0	0	0	0
	2.0	±	0	±	0	0

0 = no detectable reaction; ± = faint cloudiness without sedimentation; + = cloudiness with slight sedimentation; ++ = marked cloudiness with moderate sedimentation; +++ = marked cloudiness with large amounts of sediment.

A comparison of the responses to equal doses of the solutions which had been heated at different temperatures shows a more or less gradual diminution of the antibody-invoking property with increase in the temperature. The solution heated for 30 minutes at 55° or at 75°C. induced somewhat better responses than did the solution heated at

100°C. but the difference is less than that between the unheated solution and the solution heated at any of the test temperatures. While heating at 120°C. almost completely destroyed the antigenicity, prolonged immunization with the larger dose (2.0 cc.) invoked a weak anti-protein response.

The retention of some degree of antigenicity by the P substance of Pneumococcus after 30 minutes exposure to 120°C. is especially interesting in view of the relatively large loss in activity which follows heating at only 55°C. The antigenic effectiveness of coagulated proteins (9) involves fundamental questions which are encountered in the study of enzymes as well as in immunology, *i.e.*, whether the diminished but not destroyed response is due to a partial but common inactivation of all of the protein molecules, or to traces of active substance which remain uncoagulated at the final end-point of the coagulation reaction, or perhaps to a gradual *in vitro* or *in vivo* reversal of the coagulated protein to its original form.

Experiment 2. Influence of Heating at Different Temperatures upon the Antibody-Invoking Properties of Suspensions of Pneumococcus Cells.

The following experiment dealt with a comparison of the influence of the temperature of heating upon the antibody-invoking properties of suspensions of pneumococcus cells. In contrast to the solutions of bacterial substances studied in the preceding experiment, the suspensions of the bacterial cells contain two effective antigens: the antigenic complex (SP) which gives rise to the type-specific (anti-S) antibodies, as well as the species-specific antigen (P) which gives rise to the species-specific (anti-P) antibodies. Hence, this experiment consisted essentially in a comparison of the influence of the different temperatures upon two types of antigens (type-specific and species-specific) both of which are contained in the same pneumococcus cell.

The bacterial sediment of a centrifuged 12 hour broth culture was resuspended in one-tenth volume of salt solution, so that 1 cc. of the suspension was equivalent to 10 cc. of culture. The suspension was divided into four equal portions; the separate lots were then heated for 30 minutes at one of the following temperatures: 55°, 75°, 100°, and 120°C. Eight rabbits of approximately the same age and weight were selected and divided into two series. The individual rabbits in the first series received doses of vaccine equivalent to 0.5 cc. of culture and the individuals in the second series received doses equivalent to 5.0 cc. of culture; one animal in each series being injected with vaccine which had been heated at one of the four test temperatures. The immunization consisted of three courses of six daily

intravenous injections, with 1 week's rest between each course. 10 days after the last injection, the animals were bled, and the immune serum tested for type-specific and species-specific antibodies by the methods described.

The immune serum obtained with suspensions of pneumococci which had been heated at different temperatures (55° to 120°C.) contained the usual type-specific antibodies, regardless of the temperature at which the vaccine had been heated. However, the response of the individual animals to the different amounts of the same vaccine did not show the definite relation between amount of antigen and degree of antibody response which was evidenced in the previous experiment with the pneumococcus solutions. The serum from the rabbit injected with the smaller dose frequently contained as great an amount of type-specific antibody as did the serum obtained from animals which had received doses 10 times as large of the same material. Due to the irregularity of response and to the lack of a definite difference between the responses to the two test doses of vaccine heated at the same temperature, it was impossible to show from this experiment any general distinction between the influence of the different temperatures upon the effectiveness of the type-specific and species-specific antigens. The results as a whole, however, indicated that heating at any temperature had less effect upon the effectiveness of the type-specific antigen than upon the effectiveness of the species-specific antigen.

Experiment 3. Comparison of the Antibody (Type-Specific and Species-Specific) Response to Unheated Suspensions of Pneumococci with the Response to Heated Suspensions of Pneumococci.

The results of the preceding experiment had shown no definite difference between the responses to the pneumococcus suspensions which had been heated at different temperatures. It seemed probable that a more clean-cut distinction between the type-specific and species-specific antigens would be evidenced by limiting the comparison to the differences in the antigenic effectiveness of unheated and heated suspensions, rather than by attempting to show differences in the effect of heating at different temperatures.

In the following experiment, one series of rabbits was injected with unheated or live suspensions, and two other series were injected with suspensions heated at 55° and at 100°C. Since it was desired, if possible, to base our comparisons upon test doses which showed some direct relation between dosage and degree of response to the same lot of vaccine, it was decided to increase the difference between the test doses employed in Experiment 2, and to use for the larger dose in each series, an amount of vaccine 15 times as great as that represented by the smaller dose. The immunization was continued for four courses of injections with bleedings after the second and fourth courses. The use of two test doses and the examination of the immune serum after two and four courses of injections furnished a more valid basis for a distinction between the effect of heat upon the antigenic

effectiveness of the type-specific and species-specific antigens of the pneumococcus cell.

Twelve male rabbits of approximately the same age and weight were divided into three series. The first series of four animals received one course of injections of pneumococcus suspension which had been heated 8 minutes at 55°C. and three courses of unheated suspension. The second and third series of rabbits received four courses of the same suspension of pneumococci differing only in that the suspension had been heated for 30 minutes at 55° and at 100°C., respectively. Two rabbits in each series received daily doses equivalent to 0.4 cc. of broth culture, and the other two rabbits in the series received doses 15 times as large (equivalent to 6.0 cc. of culture). Each course of injections consisted of six daily doses with the usual period of 1 week between the first and second, and between the third and fourth courses. Bleedings were made 10 days after the last injection of the second and fourth courses, which caused an 11 day rest period between the second and third courses. Two rabbits (Nos. 25 and 29) died during the third course of injections.

The results of tests of the immune sera are collected in Table II.

The results in Table II can be analyzed as follows: After both the second and fourth courses of injections, the sera of all of the animals which had received the larger amount of vaccine contained a greater amount of type-specific antibody than did any of the animals which had received the smaller test dose. This is important, for the fact that the smaller test dose was less than that required for the maximum antigenic response furnishes a more desirable basis for a comparison of the effect of heat upon the effectiveness of the two types of pneumococcus antigens than if both test doses had been above that required for the maximum response.

If a comparison is made of the type-specific antibody responses to the larger test dose of vaccine, it is evident that no distinction can be made between the sera obtained by immunization with unheated pneumococcus cells and that obtained by immunization with suspensions which had been heated either at 55° or at 100°C. The same general relation holds true in the sera obtained by immunization with the smaller dose: although none of these sera contain as high a content of anti-S antibodies as do the sera obtained by use of the larger dose of vaccine, the use of the unheated bacterial cells did not cause a more effective response than did the pneumococci which had been heated. The results obtained with the animals immunized with the smaller doses are in one respect more convincing than that obtained

TABLE II.

Comparison of Antibody-Invoking Property of Unheated Suspensions of Pneumococcus Cells with That of Suspensions Which Had Been Heated at Different Temperatures.

Antigen used in immunization: Suspension of pneumococcus cells			Species-specific anti-protein precipitins		Type-specific antibodies							
			Serum after 2 courses of injections	Serum after 4 courses of injections	Serum after 2 courses of injections				Serum after 4 courses of injections			
					Type II agglutinins		Passive protection		Type II agglutinins		Passive protection	
Rabbit No.	Treatment of antigen	Dose in terms of broth culture	Precipitation with ½ dilution of test solution	Precipitation with ½ dilution of test solution	Dilution of serum		Dose of culture		Dilution of serum		Dose of culture	
					1/10	1/80	0.1 cc.	0.01 cc.	1/10	1/80	0.1 cc.	0.01 cc.
		cc.										
18	Unheated	6.0	+	+++	xxx	x	S	S	xxx	x	S	S
19	Unheated	6.0	+	+++	xxx	x	S	S	xxx	x	S	S
20	Heated 30 min. at 55°C.	6.0	±	+	xxx	x	S	S	xxx	x	S	S
21	Heated 30 min. at 55°C.	6.0	±	+	xxx	0	S	S	xxx	x	S	S
22	Heated 30 min. at 100°C.	6.0	±	+	xxx	x	S	S	xxx	x	S	S
23	Heated 30 min. at 100°C.	6.0	±	+	xxx	0	S	S	xxx	x	S	S
24	Unheated	0.4	±	+++	x	0	D	S	xx	0	S	S
25	Unheated	0.4	±	*	xx	0	S	S	*	*	*	*
26	Heated 30 min. at 55°C.	0.4	0	0	xx	0	S	S	xx	0	S	S
27	Heated 30 min. at 55°C.	0.4	0	0	x	0	D	S	x	0	D	S
28	Heated 30 min. at 100°C.	0.4	0	0	x	0	D	S	xx	0	S	S
29	Heated 30 min. at 100°C.	0.4	0	*	x	0	D	S	*	*	*	*

* Rabbit died during immunization.

In Tables II and III 0 = no detectable reaction; ± = faint cloudiness without sedimentation; + = cloudiness with slight sedimentation; ++ = marked cloudiness with moderate sedimentation; +++ = marked cloudiness with large amounts of sediment; x = definite granulation without formation of compact disc; xx = compact disc with faintly cloudy supernatant fluid; xxx = compact disc with clear supernatant fluid; D = mouse died in 18–60 hours; S = mouse survived 7 days.

with the larger test dose, for the fact that the test dose is known to be less than that required for the maximum response indicates that the lack of any essential difference in apparent antigenic effectiveness is not obscured by the injection of excessive amounts of total antigen.

Quite different relations are revealed in a comparison of the species-specific antibody (anti-P) response. In the case of the animals injected with the larger test dose, the sera obtained by immunization with the unheated pneumococci contain much more of the species-specific antibody than do those immunized with the heated bacterial cells. While the sera of the rabbits receiving the heated material increased in their anti-P precipitin content as the immunization was continued, it is evident that even after four courses of injections none of these animals produced more of the species-specific antibody than that invoked in other animals by two courses of injections of the unheated material. The same distinction is revealed in the results obtained in the animals which received the smaller test dose. Again, the animals which received unheated pneumococci produced more anti-P than did those which received the same amount of heated bacteria. The distinction between the anti-P response to the heated and the unheated bacterial cells is in striking contrast to the apparent lack of any difference in the anti-S response to equal doses of heated and unheated pneumococcus suspensions. Whether or not the bacterial cells have been heated is apparently the most important factor in determining the relative amount of the species-specific antibody in antipneumococcus sera, for although size of dose and length of the period of immunization also increased the anti-P response to the heated bacterial suspension, doses 15 times as great of the heated bacteria failed to invoke as effective an anti-P response as that obtained by small doses of unheated pneumococci.

These results are of additional interest as further evidence of the individuality of the type-specific and of the species-specific antigens of Pneumococcus, for the existence of two different antigens which are affected to a different extent by heat are required in order to obtain immune sera in which the relative proportion of the two antibodies is determined by the use of unheated or heated bacterial cells in the immunization.

Experiment 4. Comparison of the Antibody Response to Subsequent Injections of Unheated and of Heated Pneumococcus Cells by Rabbits Previously Immunized with Heated Pneumococci.

The following experiment differs from the preceding ones in that the animals received a preliminary immunization treatment with heated pneumococcus cells; and were then divided into two groups, one of which was given additional immunization with heated pneumococci, and the second was given additional immunization with unheated pneumococci.

It is a common practice in the preparation of antibacterial serum to employ heated bacteria in the first part of the immunization treatment and to inject live bacteria in the later stages of the immunization in the hope of increasing the protective value of the serum. Since passive protection of mice against Type II pneumococci is apparently a function of the type-specific antibody (5), the results of the preceding experiments furnished considerable ground for the belief that unheated pneumococci would be no more effective than heated bacterial cells in the production of a serum of high protective or high agglutinating value. However, in view of the possibility of differences in the responses of previously immunized animals, the following experiment was designed to compare the responses induced by subsequent immunization with unheated bacteria with the responses induced when the immunization was continued with heated bacteria alone. All of the animals selected for the final test immunization, had responded to previous immunization with the heated bacteria with the production of immune serum of high titres of type-specific antibody before the final test comparison of the effect of heat upon the bacterial cells injected in the subsequent immunization.

Eighteen rabbits were immunized with three courses of daily injections of heated vaccine, each dose being equivalent to 5.0 cc. of broth culture. The immune sera were then tested by the usual methods for type-specific and species-specific antibodies. From the original eighteen animals, twelve were selected for the final test comparison. Six rabbits which gave weak responses to the preliminary immunization were eliminated from the experiment. Each of the selected twelve rabbits had produced, in response to the preliminary immunization, sera which contained high and approximately equivalent amounts of the usual type-specific antibody (anti S precipitins, agglutinins, and passive protective action). Since the preliminary immunization consisted of eighteen injections of relatively large amounts of vaccine, it was assumed that at this stage the animals had had sufficient immunological stimulus to invoke an anti-S response approximately equivalent to the responding capacity of the individual animals. It is obvious, thus, that the following comparison of the effect of subsequent immunization with unheated and with heated bacterial cells, was limited to animals which had already given evidence of effective response to heated pneumococci and which had in fact already produced as high titres of type-specific antibody as is usually obtained in

Type II antipneumococcus rabbit serum. None of the animals had produced more than the small amount of the species-specific antibody commonly found in antipneumococcus serum of rabbits immunized with heated vaccine.

These twelve previously immunized rabbits were then divided into two series: Series *A* received heated pneumococci in the subsequent immunization; Series *B* received the same amount of unheated pneumococci; the subsequent immunization consisted of two courses of six daily injections of bacterial suspension equiva-

TABLE III.

Comparison of the Antibody Responses to Subsequent Injections of Unheated and Heated Pneumococci by Rabbits Previously Immunized with Heated Pneumococci.

Rabbit No.	Treatment of pneumococcus suspension injected in subsequent immunization	Type-specific antibody										Species-specific antibody			
		Agglutinins				Passive protection						Antiprotein precipitins			
		After 3 preliminary courses of heated pneumococcus		After subsequent immunization		After 3 preliminary courses of heated pneumococci			After subsequent immunization			After 3 preliminary courses of heated pneumococci		After subsequent immunization	
		Serum dilution				Dose of culture						Dilution of test solution			
		1/20	1/80	1/20	1/80	0.2 cc.	0.1 cc.	0.03 cc.	0.2 cc.	0.1 cc.	0.03 cc.	1/4	1/20	1/4	1/20
30	Heated	xx	x	xx	0	D	D	S	D	S	S	+	±	+	±
31	"	xx	0	xxx	0	D	S	S	S	S	S	±	0	+	±
32	"	x	0	xx	0	S	D	D	D	S	S	0	0	±	0
33	"	xx	x	xxx	x	D	S	S	D	D	S	±	0	±	0
34	"	xx	0	xxx	x	S	S	S	S	S	S	±	0	+	±
35	"	xxx	0	xxx	0	S	S	S	S	S	S	±	0	±	0
36	Unheated	x	0	xx	0	S	D	S	S	S	S	+	±	++	+
37	"	xx	0	xx	0	S	D	S	D	S	S	0	0	++	+
38	"	xx	0	xxx	x	D	S	S	S	S	S	0	0	++	+
39	"	x	0	xx	0	D	D	S	D	S	S	±	0	++	+
40	"	xx	0	xxx	x	D	D	S	S	D	S	±	0	++	+
41	"	xx	0	xxx	0	D	D	S	D	S	S	±	0	++	+

lent to 5.0 cc. of broth culture. After the end of this treatment, a second bleeding was made, and the immune serum obtained was tested by the same methods employed in the tests of the first bleeding.

The results are collected in Table III. Since the anti-S precipitins and the type-specific agglutinins proved approximately parallel in all the sera, the results of the anti-S precipitin tests are omitted in Table III.

The facts presented in Table III can be analyzed to advantage by considering first the type-specific antibody response. All of the animals in both Series *A* (heated) and *B* (unheated) had produced sera containing high titres of the type-specific antibody after the first three courses of injections. This response apparently represented the maximum response of the individual animals, since only slight increases and at times slight decreases in the content of type-specific antibody was obtained by continued immunization with either heated or with unheated pneumococci. Apparently, with the selected previously immunized rabbits used in this experiment, the slight differences that existed in the potency of the sera of the different animals was determined by the capacity of the animal to respond and was independent entirely of whether or not the bacterial cells had been heated.

This apparent total lack of any real difference between the type-specific antibody content of the sera obtained by subsequent injection of the previously immunized animals with unheated pneumococci in comparison to that obtained with heated bacterial cells was just as pronounced in passive protection experiments as in agglutination tests. All of the sera protected against about 0.1 cc. of culture and attempts to show differences by increasing the test doses failed due to the irregularities usually encountered in protection tests with doses of culture near the zone of mass infection. Other experiments were made in which a constant dose of culture (1×10^{-4} cc.) was tested against different amounts of serum. The results of these experiments showed some differences in the potency of the different sera but the differences were irregular and had no relation to the heating treatment of the immunization material.

An analysis of the species-specific antibody responses induced in the previously immunized rabbits reveals entirely different relations than those obtaining for the species-specific antibody. The sera of all the rabbits contained only small amounts of the anti-P precipitin at the end of the preliminary immunization which, as shown before, had yielded sera containing high contents of anti-S. When these previously immunized animals were subsequently injected with additional doses of heated pneumococci there was only a slight increase in the species-specific antibody yield. On the other hand, the animals which

received unheated pneumococci in the subsequent immunization treatment responded with large amounts of the species-specific antibody so that their sera after the final two test courses of injections contained anti-P precipitins in titres comparable to those obtained by immunization with large amounts of solutions of pneumococcus cells or of pneumococcus protein.

The most important facts shown by this experiment can be illustrated best by summarizing the results of Table III in the form given

TABLE IV.

Summary of the Antibody Responses to Subsequent Injections of Unheated and Heated Pneumococci by Rabbits Previously Immunized with Heated Pneumococci.

Immunization		Increase in antibody content of sera due to subsequent immunization of the previously immunized rabbits	
		Type-specific antibodies	Species-specific antibodies
Animals previously immunized with three courses of injections of heated pneumococcus cells	Animals which subsequently received two additional courses of heated pneumococci	No significant increase	No increase by 3 rabbits; slight increase by 3 rabbits
	Animals which subsequently received two additional courses of unheated pneumococci	No significant increase	Marked increase by all (6) rabbits

in Table IV. The relations evident there (Table IV) indicate that individual rabbits can produce only a certain amount of the type-specific antibody and that the maximum response can be obtained by the injection of heated pneumococcus cells; and that after this maximum responding capacity has been reached, the further injection of unheated pneumococci serves only to increase the species-specific antiprotein precipitin content without increasing significantly the type-specific antibody content of the serum.

Experiment 5. The Antihemotoxin Response of Rabbits Immunized with Heated and with Unheated Pneumococcus Cells.

Pneumococcus antihemotoxin, like the anti-P precipitin, is a species-specific and not type-specific antibody (11). The hemotoxin, the antigen which gives rise to the antihemotoxin antibody, is an endocellular substance and like the antigens which give rise to the anti-P precipitins is present in solutions of pneumococcus cells. In a previous paper (10), it was shown that the hemotoxin can be distinguished from the antigens related to the anti-P precipitins by the fact that the antibody-invoking property of the hemotoxin antigen is destroyed by heating treatment (55°C.) which diminishes but does not destroy the effectiveness of the precipitinogens. Since the hemotoxin like the P precipitinogens is contained as an endocellular constituent in the pneumococcus cell suspensions injected for the production of the usual antibacterial antipneumococcus serum, one would expect the relative proportion of the antihemotoxin contained in antipneumococcus serum to be influenced by the heating treatment of the bacterial suspensions employed in the immunization. In fact, from analogy with the results obtained in immunization with heated solutions of pneumococcus cells, one would expect the antihemotoxin content to be almost entirely dependent upon whether the bacterial cells were heated or unheated, for we failed entirely to detect any antihemotoxin in the immune serum of animals injected with heated hemotoxin solutions (10).

In order to determine the effect of the heating treatment of suspensions of pneumococcus cells upon the antihemotoxin content of antipneumococcus serum, antihemotoxin titrations were made with the sera of the forty-one animals employed in the preceding experiments. All of the sera obtained by immunization with unheated pneumococcus cells, like the sera obtained by immunization with unheated solutions of the bacterial substances, contained significant amounts of antihemotoxin. With only one exception, none of the sera obtained by immunization with heated pneumococcus cells contained any detectable amount of the antihemotoxin. The one exception consisted in the serum of a rabbit which had received prolonged immunization with large amounts of bacterial cells heated at 100°C. (Rabbit 23 in Table II). It is impossible to explain this apparent discrepancy.

In spite of the observed exception, it is logical to believe that the antihemotoxin content of antipneumococcus serum, like the anti-P precipitin content of the same sera, is determined largely by the heating treatment of the bacterial cells employed in the immunization. One would expect to find especially large amounts of this species-specific antibody in the antipneumococcus serum obtained from horses which had received large doses of live or unheated pneumococci during the later stages of the immunization.

DISCUSSION.

The preceding experiments have dealt with the influence of heat upon the antibody-invoking effectiveness of the type-specific and species-specific antigens of Type II pneumococcus cells. The results of the study of the effect of heat upon the species-specific antigen as contained in filtered solutions of the intracellular substances of pneumococci showed that 30 minutes exposure of the bacterial solutions to 55°C. or higher temperatures, caused a marked decrease in the antibody-invoking effectiveness of the species-specific antigen, not only from the standpoint of the degree of antibody response to equal doses of unheated or of heated solution, but also from the standpoint of the amount of total antigen and number of injections required for an effective response. The experiments with suspensions of pneumococcal cells were more complicated than those in which solutions were used as the immunization material, since the latter experiments included the type-specific as well as the species-specific antibodies. More differences existed in the responses of individual animals immunized with the same lot of bacterial suspension than were encountered among the rabbits immunized with the bacterial solutions. However, the large number of rabbits included, the use of different doses in the immunization, and the examination of the immune serum after different periods of immunization, furnished convincing evidence that the antibody-invoking property of the species-specific antigen as contained in the suspensions of bacterial cells was more greatly diminished by heat than was that of the type-specific antigen. For example, a series of rabbits injected with large but equal doses of heated (30 minutes at 55° or 100°C.) or of unheated suspensions of pneumococci, contained in common a high content of type-specific antibody independent of the heating treatment of the antigen: and in contrast to their common content of the type-specific antibody, the same sera contained large amounts of the species-specific antibody only where unheated bacteria had been employed in the immunization. Moreover, a comparison of the sera of another series of rabbits showed that the immune serum obtained by injection of small amounts of unheated pneumococcus suspension (below the dose required for the most effective response) contained less type-specific but more species-

specific antibody than did the serum of other rabbits which had received doses 15 times as great of the heated bacterial cells. The production of immune serum in which the relative proportion of type-specific to species-specific antibody seems to be directly dependent upon whether or not the immunization material had been heated or unheated, presents convincing evidence of differences in the effect of heat upon the antibody-invoking effectiveness of the type-specific and species-specific antigens of Pneumococcus. It is important to observe that these relations were established in experiments in which dosage and length of time of immunization were carefully controlled, for it would be theoretically possible to obtain similar responses to heated and unheated antigen if the total amount of antigen were greatly in excess of the dose required for the maximum immunity response.

The species-specific antigen of Pneumococcus is endocellular and it can be assumed that the bacterial cells must be dissolved, either before or after injection into the animals, in order to liberate the antigen and permit it to come into direct contact with the cells involved in antibody production. Since unheated pneumococci autolyze *in vitro* more readily than do the heated bacterial cells and are likewise more soluble in bile, the question might be raised whether a greater rate of lysis of the unheated cells *in vivo* may not be a factor in the greater effectiveness of the endocellular antigen. But *in vivo* and *in vitro* dissolution of pneumococci are distinctly different processes: one of them due in large part to the specific heat-labile bacteriolytic enzyme produced by the bacteria themselves (12) and the other to lytic action of the cells or fluids of the animal. However, the possibility of differences in the ease of lysis of the unheated and heated pneumococci, while not to be ignored, does not seem to be an important point for the fact that the antibody-invoking effectiveness of the protein as contained in the solutions employed in the first experiment was influenced as markedly by heat as was the protein contained within the undissolved cells in later experiments, indicated that the decrease in antigenic activity represents a real change in the bacterial protein which occurs whether heated inside or outside the bacterial cell.

The distinct difference in the relative effect of heat upon these two antigenic constituents of the pneumococcus cell is of interest from both theoretical and practical points of view. It is important to note that

our experiments have dealt with the antibody-invoking and not with the antibody-combining property of the two antigens. The type-specific S substance when free in solution, dissociated from the antigenic complex SP, does not possess the antibody-invoking property whether heated or unheated, but it retains its antibody-combining property after even the most drastic heating treatment (in approximately neutral solutions). The resistance of the antibody-combining property of the S substance to the autoclaving and boiling in the open water bath, both of which are preliminary steps in the preparation of the purified substance (1, 2), is an outstanding example of heat stability among immunologically reactive substances. However, even the antigenic complex itself (SP), undissociated and in the antibody-invoking effective form, is relatively heat-resistant in comparison to many antigens of bacterial origin and, from the antibody-invoking standpoint is decidedly more heat-stable than the species-specific protein antigens contained within the same pneumococcus cell. Possibly, combination with the carbohydrate or S substance endows pneumococcus protein with new physical properties (resistance to heat coagulation) as well as conferring upon it the new chemical or immunological properties which are responsible for type specificity.

The difference in the heat lability of the type-specific and species-specific antigens of Pneumococcus must be an important factor in determining the relative proportion of the type-specific and species-specific antibodies in all antipneumococcus serum produced by injection of pneumococcus suspensions. While the type-specific antibody (anti-S) is the predominant and important one, a certain amount of the species-specific antibody accompanies it in practically all type-specific sera. The species-specific antibody content and the ratio between type-specific and species-specific antibody vary in sera produced in different laboratories; one serum may contain relatively large amounts of the species-specific antibody and another may contain only difficultly detectable traces of the same antibody, even though the type-specific antibody content of the two sera is approximately identical. The factors which operate in determining the relative amount of species-specific antibody must include those which determine the degree of the response to any antigen: treatment of the immunization material, dosage, period of immunization, and the re-

sponding capacity of the individual animal. However, if the vaccine is prepared from virulent, S-producing pneumococci and reasonable doses are injected in a course of immunization not unnecessarily long, one could expect that heating of the pneumococcus suspension injected would greatly decrease the production of species-specific antibodies (anti-P) without an appreciable effect upon the production of type-specific antibodies (anti-S). This is shown to be true with rabbits in the preceding experiments.

The results of the experiments on subsequent immunization of rabbits previously immunized with heated pneumococci, indicated that the only thing gained by the subsequent immunization is an increase in the species-specific antibody content of the serum. Since this latter antibody is not involved in truly type-specific agglutination, the conclusion may be drawn that continued immunization of animals which already have responded with high titres of type-specific antibody adds nothing at all to the diagnostic value of the serum. The use of live or unheated bacteria is unnecessary in order to produce a highly potent type-specific sera, and, if the sera are to be used for typing, the increase in species-specific antibody without increase in the type-specifically reacting substances is undesirable.

SUMMARY.

This paper presents an experimental comparison of the effect of heating of the immunization material upon the antibody-invoking effectiveness of the type-specific (SP) and species-specific (P) antigens of Type II pneumococci. Heating of the pneumococcus suspension (vaccine) invariably decreased the production of species-specific antibodies (anti-P) without a comparable effect upon the production of type-specific antibodies (anti-S).

For diagnostic typing purposes, the ideal antipneumococcus serum should contain the maximum content of type-specific, and the minimum of species-specific antibody. Our results with forty-one rabbits indicate that the ideal serum from the type-specific standpoint would be obtained by immunization with the heated cells of virulent pneumococci over a comparatively short immunization period; and that the only thing gained by continued immunization or by the use of unheated bacteria at any stage of the immunization, is an increase in

the species-specific antibody which is undesireable in sera to be used for diagnostic purposes.

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STUDIES ON STREPTOCOCCUS BACTERIOPHAGE.

II. The Influence of Lytic Principles upon the Agglutination of Hemolytic Streptococci.

By GREGORY SHWARTZMAN, M.D.

(*From the Laboratories of the Mount Sinai Hospital, New York.*)

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INTRODUCTION.

Bacteriophage is capable of inducing considerable disturbance in susceptible bacterial cells. Especially interesting is the influence of this principle upon the agglutinative properties of bacteria. The studies on the subject reported by d'Hérelle (1), Bordet and Ciuca (2), Gratia (3), Flu (4), McKinley (5) and others were limited to the *coli*-typhoid-dysentery group of bacteria. The observed changes consisted of loss of agglutinability by specific sera with and without loss of agglutinogenic and agglutinin-absorbing properties. In one instance, as reported by Gratia (3), the effect of the lytic principle consisted in appearance of agglutinability in a previously inagglutinable strain of *B. coli*.

The author's object was to extend these studies to other bacteria in which the bacteriophage phenomenon was shown to exist. The hemolytic pathogenic streptococci were chosen for this work for the following reasons:

1. It was shown by Dutton (6) that many strains of human pathogenic streptococci existed symbiotically with phage of low potency. Two strains of human origin were shown to undergo lysis (Piorkowski (7) and Dutton (6)). The author demonstrated the classical bacteriophage phenomenon among several strains of erysipelas origin (8). Since, therefore, it can be safely accepted that the bacteriophage phenomenon exists among streptococci, it seemed possible that there might exist a definite influence of phage upon the phenomenon of agglutination of these microorganisms.

2. The complexity of the problems of serological specificity and affiliations of different groups of streptococci makes it important to determine the influence of the phage which represents a powerful factor in the life of many species of bacteria, including streptococci.

The plan of this work was influenced by the following considerations:

Since the effect of the phage upon agglutination of streptococci is entirely unknown it was decided to study the effect of the phage upon the entire strain. Later should a definite relation be established, these studies could be extended to strain components derived from single colonies or single cells.

In view of the fact that various strains of pathogenic streptococci differ in the degree of susceptibility to the phage it was presumed that the changes induced would vary as well. For this reason a large number of strains was employed.

Since various groups of hemolytic streptococci differ in the complexity of the antigen it was also thought that the same agent would induce different changes, the nature of which might depend upon the antigenic structure. This consideration led to the study of representatives of various serological groups of streptococci.

Moreover, since lytic principles from various sources differ in their influence upon streptococci (Shwartzman (8)) it was decided to limit the work to the study of one or two lytic principles and employ these throughout the work.

In order to be able to obtain a full conception of the various possible changes which this principle may induce in streptococci under the conditions outlined above, the studies were made first by means of direct *agglutinations*. The information gained was further substantiated by studies on *the agglutinin-absorbing and agglutinogenic properties* of each culture under discussion. To study the effect of phage upon the affiliations between various streptococci each serum was tested against the majority of strains on hand.

Methods.

Lytic Principles.—SL bacteriophage, active against a streptococcus from rabbit's lung and also potentially active against streptococci of erysipelas origin; the C/2, anti-*B. coli* phage were used in these experiments. Cultures of various streptococci were grown in phosphate broth containing 1 to 10 dilution of lytic

principle for a number of generations. These cultures will be referred to as SL and C/2 respectively. *The strains* employed in this work were as follows:

1. The erysipelas group, represented by ten strains kindly sent to me by Dr. Konrad Birkhaug under the name E_1-E_{10}. All these strains were potentially susceptible to SL lytic principle only.

2. Scarlet fever strains (55, 108), sent to me by Dr. A. W. Williams, which were found resistant to both lytic principles.

3. A strain of *pyogenes* hemolytic streptococcus, isolated in this laboratory from a case of meningitis and called H.S. This was resistant to both principles.

4. A strain of green-producing streptococcus, isolated from the blood of a case of subacute bacterial endocarditis, under the name V. This was also resistant to the lytic principles employed.

5. Rb streptococcus, a pyogenic hemolytic strain isolated by Clark and Clark from rabbit lung infection. This streptococcus was highly susceptible to SL bacteriophage and resistant to C/2 lytic principle.

Immunization.—Two rabbits were immunized with each strain and the serum of higher titer was selected for the work. The method of immunization was that recommended by Dochez (9).

Agglutination.—Homogenous antigens were obtained by growing streptococci in 0.1 per cent glucose phosphate broth. Phage strains were passed through phosphate broth containing 1 to 10 dilution of lytic principle. Both normal and phage cultures were subcultured into phosphate broth and used for agglutination after 24 hours incubation. A considerable number of experiments was first performed with the washed sediment of these cultures. The process of centrifuging, however, brought about frequent spontaneous agglutination of previously homogenous antigens. This procedure was then given up. Care was always taken to use cultures not older than 24 hours. Parallel experiments did not show any difference in agglutination of washed and unwashed *24 hour* old cultures. No formaldehyde was added to the antigens. Serum dilutions were made in phosphate broth. Readings were made after 4 hours incubation in a water bath at 55°. The agglutinations registered in the protocols were examined by the naked eye.

Absorption of Agglutinins.—Bacterial cells for absorptions of agglutinins were prepared by centrifuging and washing the growth of 24 hour old broth cultures. A dose sufficient to absorb completely agglutinins from the homologous serum was used. In making comparative studies on the agglutinin-absorbing power of various antigens the quantitative relationship of absorbing cells to the amount of serum (usually diluted 1 to 10) was kept in mind. The mixture of given amounts of cells (heated previously to 60°) and serum was kept in the water bath at 37° for 2 hours and in the refrigerator overnight. In order to titrate the absorbed sera, dilutions from 1 to 20 up to 1 to 25,000 were always made. Serum diluted 1 to 10 but unabsorbed, to be used for control titration, was kept under the same temperature conditions as the serum for absorption.

The results reported below were grouped according to the changes produced by given lytic principles in the various strains of pathogenic streptococci.

EXPERIMENTAL.

I. Inagglutinability and Partial Loss of Agglutinogenic and Agglutinin-Absorbing Properties of "Whole" Cultures.[1]

Strain 55 which represented serologically a large group of scarlet fever streptococci was cultured for forty passages in phosphate broth and in phosphate broth containing SL phage. The effect of the phage is shown in Table I.

TABLE I.

The Influence of Phage upon Agglutination of Strain 55.

Sera	Absorbed by strain	Agglutination titer before absorption and percentage of absorption	
		55	55 SL
I	=	6400	0
I	55	100 per cent	—
I	55 SL	50 per cent	—
II	=	400	0
III	=	800	0

0, no agglutination; = no absorption; — not tested.
I, 55 strain serum; II, 55 SL strain serum; III, 55 SL strain serum.

As is seen the phage strain was rendered completely inagglutinable by normal culture serum. This change was coincident with partial loss of agglutinin-absorbing properties and also with what appeared a certain loss in the agglutinogenic power. In fact the immunization of two animals with the phage cultures resulted in a distinctly lower titer of agglutinins. These sera agglutinated the normal culture but did not react with the homologous culture.

Similar but somewhat more marked changes were obtained in Rb streptococcus.

[1] The change demonstrated in this work occurred in entire cultures. It remains yet to determine whether this was due to actual changes in the cells bearing the agglutinogens or to simple elimination of these cells by means of the lytic agent.

This streptococcus highly susceptible to SL phage was made resistant to this principle by means of several passages through broth containing at first 1×10^{-6} cc. and later 1×10^{-1} cc. dilution of the phage. Observations on cross-agglutinations and cross-absorptions of sera prepared by immunization with normal Rb streptococcus, the resistant type and lysed culture of the same strain are given in Table II. As is seen, Rb streptococcus was considerably affected by passage through SL phage. The strain became inagglutinable by the normal culture serum as well as by heterologous sera which normally cross-

TABLE II.

The Influence of Phage upon Agglutination of Rb Streptococcus.

Sera	Absorbed by strain	Agglutination titer before absorption and percentage of absorption			
		Rb streptococcus	Resistant Rb strepto-coccus	55	130
IV	=	6400	0	400	40
IV	Rb streptococcus	100 per cent	—	0 per cent	0 per cent
IV	Resistant Rb. streptococcus	0 per cent	—	—	—
V	=	50	0	0	0
VI	=	0	0	0	0
VII	=	0	0	0	0
VIII	=	0	0	0	0
IX	=	0	0	0	0
Ia	=	200	0	5120	400
X	=	200	0	—	3200

IV, Rb strain serum; V, VI, VII, lysed culture Rb strain serum; VIII, IX, resistant Rb strain sera; X, 130 strain serum (55 and 130 belong to the scarlet fever group); Ia, 55 strain serum.

agglutinated with this strain. The i gglutinability was coincident with considerably more marked changes of the antigen than those shown in the previous experiments. There was a very significant loss of agglutinin-absorbing power on the part of the resistant type and also what appeared as an entire loss of agglutinogenic properties in both lysed and resistant cultures. Five animals failed to respond to repeated immunization with these antigens. Additional attempts to immunize with such a strain are necessary before complete loss of the agglutinogenic properties can be safely accepted.

It should be pointed out that the group components of this strain underwent similar changes. In contrast to the normal Rb culture the resistant streptococcus failed to agglutinate with Strains 55 and 130 and also failed to stimulate the production of group agglutinins for the two strains.

II. Inagglutinability with Complete Preservation of Agglutinogenic and Agglutinin-Absorbing Properties. Appearance of Additional Components.

Examples of partial modification of antigens under the influence of phage were afforded by representatives of various groups of streptococci:

A. The Erysipelas Group of Streptococci.—Table III represents the results of direct agglutinations of normal and phage cultures of erysipelas strains. As is shown by Birkhaug (10), Dochez (9) and Tunnicliff (11) this group of streptococci possesses a specific antigen. In this work the serum for one of these strains agglutinated to a high titer several representatives of this group. The bacteriophage had a definite effect upon these strains. SL lytic principle, potentially active against these strains, lowered the agglutinability of eight strains out of ten tested. This change, however, did not alter the specific component of the strains since the agglutinin-absorbing power of all the phage cultures remained unaffected. The specific agglutinins of Serum XI were completely absorbed by these cultures (not recorded in the tables). Moreover, Serum XII agglutinated the normal strains to a high titer, as is seen from Table III. However, another component was rendered prominent in the phage cultures, as evidenced by the following observation. The phage culture serum (XII) agglutinated all the phage cultures to a higher titer than the normal cultures when tested soon after bleeding (not given in Table III). Since these strains agglutinated to a lower titer with the normal culture serum it suggested that another component was rendered prominent in the phage cultures.

This assumption was further strengthened by another observation. As is seen from Table III, when sterile, non-preserved Serum XII was retested 3 weeks after bleeding, the phage cultures this time agglutinated to a lower titer than the normal cultures. A new phage culture serum (XIII) was then prepared and a part of it was added with

TABLE III.

The Influence of Phage upon Agglutination of Erysipelas Streptococci.

Agglutination Titer.

Sera	E_1	SL E_1	E_2	SL E_2	E_3	SL E_3	E_4	SL E_4	E_5	SL E_5	E_6	SL E_6	E_7	SL E_7	E_8	SL E_8	E_9	SL E_9	E_{10}	SL E_{10}	55	SL 55	108	SL 108	Rb
XI	6400	200	12800	800	3200	0	6400	1500	800	800	12800	1600	3200	0	6400	400	12800	1600	25600	25600	0	0	0	0	0
Sterile non-preserved; tested 3 wks. after bleeding XII	1600	200	1600	400	1600	400	3200	200	800	0	1600	400	1600	0	1600	0	1600	0	3200	3200	0	0	0	0	0
3 wks. after bleeding; phenolized XII	6400	12800	6400	12800	3200	6400	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2 wks. after bleeding; phenolized XIII	800	1600	800	6400	1600	6400	800	3200	800	800	800	3200	200	1600	800	800	400	3200	400	400	—	—	—	—	—
Sterile non-preserved immediately after bleeding XIII	800	1600	800	6400	1600	6400	800	3200	800	800	800	3200	200	1600	800	800	400	3200	400	400	—	—	—	—	—

XI, E_1 normal culture serum; XII, E_1 SL phage culture serum; XIII, E_1 SL phage culture serum.

phenol.[2] The new serum, sterile but not preserved, when tested the following day after bleeding showed agglutination of a higher titer with phage cultures. Part of Serum XII, to which phenol was added immediately after bleeding when tested 3 weeks after bleeding also showed higher agglutination with the phage cultures. Serum XIII, preserved with phenol, titrated 2 weeks after bleeding, showed the same titer as the same serum non-preserved but tested immediately after bleeding. It became thus evident that the phage culture stimulated production of agglutinins specific to all the phage cultures. These agglutinins were of unstable nature and unless the serum was preserved with phenol, they soon disappeared. The relation of this additional component to the specific erysipelas component was established by cross-absorption. Four normal cultures absorbed completely all the agglutinins from phenolized Sera XII and XIII. Each of four phage cultures employed absorbed agglutinins for all the phage cultures. It was clear that the additional phage culture component was closely related to the specific erysipelas streptococcus antigen and that the phage culture components of various erysipelas streptococcus strains were closely affiliated as well.

B. The Scarlet Fever Group of Streptococci.—Strain 108 was cultivated for twenty-two passages in phosphate broth and in phosphate broth containing SL phage. The phage culture was studied as shown in Table IV. As is seen, Strain 108 became under the influence of phage relatively inagglutinable by the normal culture serum (XIV). This change, however, was not coincident with loss of agglutinin-absorbing and agglutinogenic properties, since, 108 phage culture completely absorbed Serum XIV and also stimulated production of agglutinins for 108 normal culture. Parallel to the inagglutinability of the specific 108 component another antigen appeared in the phage culture. This was evident from the fact that Serum XV, in contrast to Serum XIV, agglutinated the phage culture as well as the normal culture of Strain 108. Since, however, the normal 108 culture was able to absorb completely agglutinins for the phage culture from Serum

[2] All the sera including Serum XII were collected under sterile precautions by heart puncture and not preserved by phenol. Then the animals were killed, the blood clot removed and its serum preserved with phenol.

XV it was concluded that the phage culture component was closely related to the 108 normal culture specific antigen.

To complete the investigation of the phage culture component, its relation to another strain of the scarlet fever group was studied by cross-agglutinations and cross-absorptions. Strain 55, chosen for this purpose, had common components with Strain 108 in addition to a heterologous antigen (Table IV). It was then observed that 108 phage culture serum agglutinated Strain 55 to a far higher titer than that shown by 108 normal culture serum. Normal culture 55 serum

TABLE IV.

The Influence of SL Phage on Agglutination of 108 Strain.

Sera	Absorbed by strain	Agglutination titer before absorption and percentage of absorption		
		55	108	108 SL
XIV	=	400	3200	200
XIV	55	100 per cent	0 per cent	0 per cent
XIV	108	100 per cent	100 per cent	100 per cent
XIV	108 SL	100 per cent	100 per cent	100 per cent
XV	=	1600	1600	3200
XV	55	100 per cent	0 per cent	0 per cent
XV	108	100 per cent	100 per cent	100 per cent
XV	108 SL	100 per cent	100 per cent	100 per cent
I	=	6400	400	3200
I	55	100 per cent	100 per cent	100 per cent
I	108	50 per cent	100 per cent	100 per cent
I	108 SL	25 per cent	100 per cent	100 per cent

XIV, 108 normal culture serum; XV, 108 SL phage culture serum.

agglutinated the 108 phage culture to a considerably higher titer than the normal culture of the same strain. It became, therefore, apparent that the phage culture 108 components belonged to the "group" variety. However, Strain 55 absorbed agglutinins for itself but failed to absorb agglutinins for 108 phage culture from Serum XV. It had to be assumed that the phage components were of a more complex structure than the 55 strain group antigen.

The conclusion to be drawn was that the phage was able to render prominent additional components of a complex antigenic structure. These components were partially of the "group" variety and, there-

fore, made possible a certain measure of agglutination with a heterologous strain.

C. Rb Streptococcus.—Another example of partial modification was brought out by Rb streptococcus which was cultivated in C/2 phage. It should be pointed out that the strain was completely resistant to this phage. Table V represents the results obtained. As is seen, here again Rb streptococcus C/2 culture became inagglutinable by the normal culture serum. The inagglutinability was not coincident with any changes in agglutinin-absorbing and agglutinogenic proper-

TABLE V.

The Effect of C/2 Phage upon Agglutination of Rb Streptococcus.

Sera	Absorbed by strain	Agglutination titer before absorption and percentage of absorption		
		Rb streptococcus	Rb C/2 strain	V
XVI	=	6400	0	0
XVI	Rb streptococcus	100 per cent	—	—
XVI	Rb C/2	85 per cent	—	—
XVI	V	0 per cent	—	—
XVII	=	0	1600	3200
XVII	Rb streptococcus	—	0 per cent	0 per cent
XVII	Rb C/2	—	100 per cent	100 per cent
XVII	V	—	100 per cent	100 per cent
XVIII	=	1600	800	800
XVIII	Rb streptococcus	100 per cent	0 per cent	0 per cent
XVIII	Rb C/2	100 per cent	100 per cent	100 per cent
XVIII	V	0 per cent	100 per cent	100 per cent

XVI, Rb streptococcus serum; XVII, V strain serum; XVIII, Rb C/2 strain serum.

ties since Serum XVIII showed agglutinins for Rb streptococcus and the Rb C/2 strain absorbed agglutinins from Serum XVI for the normal culture of this streptococcus. The change was, however, accompanied by the appearance of another component in the Rb C/2 strain, since its serum, in contrast to the normal Rb streptococcus serum, agglutinated the homologous as well as the normal culture to a high titer. The additional component of the phage culture was not related to the specific Rb streptococcus antigen, as judged from the inability of the Rb streptococcus to absorb agglutinins for Rb C/2 strain from Serum XVIII.

To investigate the nature of the additional phage components Serum XVIII was tested against several strains of streptococci. This serum was able to cross-agglutinate with a strain of green-producing streptococcus. No cross-agglutination was obtained with any hemolytic streptococci tested (the negative findings are not given in Table V). The green-producing streptococcus serum was then found to agglutinate the Rb C/2 strain. Moreover, cross-absorption experiments established the very close similarity of the V specific component with the additional phage culture components. In this case the added component was of the "group" variety. In the case of scarlet fever streptococci a similarly arising component was found related only to the "group" component of another strain of scarlet fever streptococci. In the present example, however, the phage component was related very closely to the specific antigen of a strain of heterologous streptococci.

III. Transformation of the Normal Culture Agglutinogens into Antigens of an Entirely Different Specificity.

As is seen from Tables VI and VII, H.S. and V streptococci when treated with SL phage for a number of generations underwent an even more striking modification. The changes consisted of complete disappearance of normal antigen and appearance of a different antigen, which had the power of absorption, agglutinability and agglutinin-stimulating properties characteristic of a new specificity. The changes described here were in contrast to those of the first category reported above[3] and were of a more marked nature than those described under Paragraph II.

Before concluding this paper it was necessary to investigate whether the phenomenon of paragglutination played any rôle in the observations described above. For this purpose the following experiments were made.

1. The lytic principles employed in this work also contained products of bacteria, at the expense of which the phages were regenerated. It was decided to determine whether bacteriophage-free filtrates of these cultures would not induce similar changes. Filtrates of 1 week old

[3] Page 154.

Rb streptococcus and *B. coli* 42 were used. Several passages of 55, 108 and V strains were made in phosphate broth containing 1 to 10 dilutions of Rb streptococcus filtrate. Rb streptococcus was passed through *B. coli* 42 filtrate. Agglutination reactions of the cultures prepared in this manner with homologous normal culture sera did not differ from those obtained with homologous cultures. It thus became

TABLE VI.

The Influence of SL Phage upon Agglutination of V Strain.

Sera	Absorbed by strain	Agglutination before absorption and percentage of absorption	
		V	VSL
XVII	=	3200	0
XVII	V	100 per cent	—
XVII	V SL	0 per cent	—
XIX	=	0	5120
XIX	V	—	0 per cent
XIX	V SL	—	100 per cent

XIX, V SL strain serum.

TABLE VII.

The Effect of SL Phage upon Agglutination of H.S. Strain.

Sera	Absorbed by strain	Agglutination titer before absorption and percentage of absorption	
		H.S.	H.S./SL
XX	=	0	1280
XX	H.S.	—	0 per cent
XX	H.S. SL	—	100 per cent
XXI	=	5120	0
XXI	H.S.	100 per cent	—
XXI	H.S. SL	0 per cent	—

XX, H.S. SL phage serum; XXI, H.S. serum.

apparent that the phenomenon of paragglutination was not responsible for the changes described in this paper. This belief was further strengthened by the following additional observations.

2. Three animals immunized with SL and C/2 lytic principles failed to show agglutinins for the bacterial strains from which these principles were derived.

3. Sera prepared with SL phage cultures of various strains cross-agglutinated with Rb streptococcus only in those cases in which the normal culture sera cross-agglutinated as well.

4. Various strains of streptococci (erysipelas and scarlet fever groups) which normally cross-agglutinated with Rb streptococcus failed to do so when passed through SL phage.

5. Rb C/2 and V strains were not agglutinated by the anti-C/2 serum.

6. Rb C/2 strain serum failed to agglutinate *B. coli* 42.

CONCLUSIONS AND SUMMARY.

A series of experiments was carried out on various strains of streptococci in order to ascertain the changes which bacteriophage produces in the phenomena of agglutination of these organisms. The results can be placed in the following categories.

1. Loss of specific agglutinability was observed with partial and with what appeared as complete loss of specific agglutinin absorption and agglutinogenic properties.

2. Partial modification of the antigen, bringing about inagglutinability of the strains with complete preservation of agglutinogenic and agglutinin-absorption properties. Coincidentally, additional components appeared. Some of these were related to the specific antigens of the organisms from which they were derived, while others were not. In two instances these components were of "group" character.

3. "Complete" modification observed with two strains consisting of complete transformation of the normal culture agglutinogens into antigens of an entirely different specificity.

It appears from these studies that the bacteriophage phenomenon may play an intricate rôle in serological grouping of various strains of pathogenic streptococci.

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ETIOLOGY OF OROYA FEVER.

IX. Bacterium peruvianum, N. Sp., a Secondary Invader of the Lesions of Verruga Peruana.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 6.

(Received for publication, November 3, 1927.)

In the course of an experimental investigation of Carrion's disease, at the stage when bacteriological and experimental study of the characteristic lesions of verruga peruana was desirable, I was so fortunate as to obtain, through the kindness of Professor Oswaldo Hercelles, of Lima, two subcutaneous nodules, excised under aseptic conditions, from two verruga patients (Cases P 5 and J 45), and forwarded in separate sealed tubes in the ship's refrigerators from Lima to New York. A strain of *Bartonella bacilliformis* was isolated from one of the nodules (Case P 5), as has been reported elsewhere,[1] but another microorganism was also present in the tissue, and was isolated in pure cultures in both instances. This organism is particularly interesting because of its striking morphological similarity to *Bartonella bacilliformis* and its marked difference from this parasite in cultural and pathogenic properties.

Cultural Properties.

The microorganism in question grows rapidly on ordinary bacteriological culture media at 37°C. as well as at 25°C., the latter temperature being the optimum one. Round, discrete, elevated, grayish, translucent colonies measuring 1 to 2 mm. in diameter make their appearance on a plain or blood agar plate within 24 hours. They reach a size of 2 to 4 mm. within several days but seldom become much larger. The colonies are spherical, and the margin smooth; in consistency they are somewhat firmer than colonies of *B. coli* or *B. proteus*, and they show no

[1]Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

tendency to spread over the surface of the medium. In 7 to 10 days at room temperature a trace of brownish tint appears in colonies on the surface of agar. On blood agar plates hemolysis takes place and gradually extends to a considerable distance around the colonies. A characteristic pungent odor is noticeable when the plate is uncovered. Broth is diffusely clouded by the growth of the organism, and within a few days a delicate grayish pellicle is formed around the wall of the culture tube at the top of the fluid and a fluffy sediment at the bottom. As the culture grows older the broth becomes markedly mucilaginous. No putrefactive or other odor is produced either in broth or on plain agar. Löffler's serum undergoes slow but complete liquefaction within about 2 weeks at 25°C. No carbohydrates are fermented by the organism, and no hydrogen sulfide is produced on any medium. Cultures remain transplantable for a period of at least 3 months if kept constantly at 25°C. No growth takes place under strictly anaerobic conditions.

Morphology.

Individuals in young colonies on an agar surface are actively motile and measure about 0.3 to 0.4μ in width and 0.6 to 1.2μ in length. In older cultures longer forms, 2 to 4μ, are present. When grown on a blood agar slant or on leptospira medium the organisms are somewhat thinner (0.2 to 0.3μ in width) and longer than the young forms on an agar surface and give the impression of delicate slender rods (Fig. 3). They are less actively motile and may be clumped into masses of many individuals (Fig. 1) which resemble to some extent the characteristic masses of *Bartonella bacilliformis*. Forms grown on broth are of medium size and remain actively motile for many days. In cultures several days old the motile organisms are intermingled with small oval or coccoid forms which may be in pairs or short chains. Except for occasional very long individuals, which may be bent and thickened at one end, the organisms are as a rule straight and have pointed ends. No bifid or branching individuals have so far been noted.

The organism is Gram-negative and stains fairly well with fuchsin or methylene blue. Brief staining in Giemsa's solution gives it a lavender color; with more prolonged staining it becomes reddish. The general aspect of the organism in stained smears (Figs. 1 and 2) resembles that of *Bartonella bacilliformis* in similar preparations, but the individual organisms are more sharply defined than are the individual bartonellas, owing perhaps to a greater affinity for the dyes. No tendency to bipolar staining has been noticed.

One of the striking features of the organism is its characteristic spirally curved flagella (Fig. 4) which are present at one end of the body and may be two, three, or four in number. So far as I am aware, the only other pathogenic organism having flagella of this type is *Bartonella bacilliformis*.

Because of its presence in material derived from Peru, the organism has been named *Bacterium peruvianum.*

Pathogenicity.

When *B. peruvianum* is inoculated intradermally into monkeys (*Macacus rhesus*, *Cercopithecus callitricus*) or dogs, the sites of inoculation become edematous, congested, and necrotic within a few days, and open ulcers with raised margins finally result; these usually heal within 10 to 14 days. The Strain P 5 was more actively pathogenic than the J 45. Unbroken nodules such as were occasionally produced by the P 5 strain resolved after about 10 days.

Cultures made from the blood of the animals during the height of the local reactions remained sterile in all instances; there was no invasion of the general circulation by the organism. Whether or not the injection of *B. peruvianum* into the blood circulation in large quantities will result in fatal septicemia has not been determined.

B. peruvianum induces an acute fatal septicemia in rabbits, guinea pigs, rats, and mice, when administered intravenously or intratesticularly. A broth culture 48 to 72 hours old was lethal within 24 to 48 hours in a quantity of 0.1 to 1 cc. Autopsy reveals very striking and characteristic changes in the abdominal viscera.

The liver seems to be most affected; it is somewhat enlarged, tense, uniformly peppered with innumerable minute, intensely red spots which make the whole organ appear red. The stomach shows many diffuse and punctiform hemorrhages recognizable through the congested serosa. The entire intestine appears diffusely limpid red. The kidneys are swollen and congested, and the adrenals are much enlarged and deep red. The spleen is not noticeably enlarged but is soft and extremely dark bluish red. The peritoneal cavity contains some limpid pink fluid. No fibrinous exudate was found on the surface of any organ. The abdominal muscles are frequently spotted with hemorrhagic areas of moderate size. The lungs are congested but show no hemorrhages. The heart is flabby, but there is no pericarditis or pleurisy. *B. peruvianum* can be recovered in pure culture from heart's blood or spleen.

When inoculated intratesticularly, the animals succumb within 1 to 3 days and show the same changes as do animals injected intravenously. The scrotum and testicle both show intense edema, congestion, hemorrhages, and necrosis.

There was no intercurrent infection with *B. lepisepticus* or *B. monocytogenes*[2] in any of the animals.

Differentiation of B. peruvianum from Other Pathogenic Microorganisms.

The characteristics of *B. peruvianum* may be summarized as follows: Proper motility by means of multiple unipolar flagella; non-retention of Gram's stain; strict aerobiosis; better growth at 25°C. than at 37°C.; presence of proteolytic and absence of sugar-splitting ferment; mucin production in broth; hemolytic action; and a wide range of characteristic pathogenic properties for rabbits, guinea pigs, rats, mice, dogs, and monkeys. No microorganism having been previously described which corresponds with the foregoing description, the organism in question is regarded as a new species.

Bartonella bacilliformis resembles *B. peruvianum* in morphology, in its inability to ferment carbohydrates, in growing better at 25°C. than at a higher temperature, in not retaining Gram's stain, in being strictly aerobic, and in having one to four unipolar flagella. The two organisms differ widely in other respects, however. *Bartonella bacilliformis* is unable to grow on any fluid medium so far tried, or any medium which does not contain blood elements; it grows very slowly, and the colonies are very minute; it lacks proteolytic or hemolytic activity, it is difficult to disperse into a homogeneous suspension, and above all, it has specific pathogenic properties. Moreover, a potent anti-*bacilliformis* immune serum gives a specific complement fixation and a distinct agglutination reaction with *Bartonella bacilliformis*, but none with *B. peruvianum*.

B. proteus and allied organisms are distinct from *B. peruvianum* in morphological and cultural properties. The flagella of the *proteus* group are peritrichal, and hydrogen sulfide is produced by the organisms in the course of growth.

The *coli-enteritidis-typhoid* group ferments carbohydrates, and does not liquefy Löffler's serum. The flagella of this group are peritrichal.

[2] Murray, E. G. D., Webb, R. A., and Swann, M. B. R., *J. Path. and Bact.*, 1926, xxix, 407. Pirie, J. H. H., *Pub. South African Inst. Med. Research*, 1927, iii, 185. I am indebted for cultures of this microorganism to Dr. J. C. G. Ledingham, Director of the National Collection of Type Cultures at the Lister Institute.

B. monocytogenes has a single polar flagellum and the property of fermenting a great many carbohydrates.

Bacillus rickettsiformis[3] resembles *Bacterium peruvianum* in some respects, in morphological features, in growing best at low temperatures, and in producing mucin in broth cultures But the *rickettsiformis* is non-pathogenic and ferments several sugars.

SUMMARY.

A minute, pleomorphic, motile, Gram-negative bacterium has been isolated from two specimens of nodular tissue from human verruga. In films and sections of the original tissues the organism in question is difficult to distinguish from *Bartonella bacilliformis*, with which it was associated, and even in pure culture it has a number of properties in common with that parasite. No sugars are fermented by it, it is an obligate aerobe, the optimum temperature for its growth is 25°C., and it has two to four spiral flagella attached to one end of the body. It is, however, readily cultivated on any ordinary culture medium. Broth cultures contain much mucin, but no hydrogen sulfide is formed. Coagulated serum is liquefied by its growth, and the red corpuscles in a blood agar plate are hemolyzed.

Rabbits, guinea pigs, rats, and mice develop acute, fatal septicemia as a result of intravenous or intratesticular inoculation of young cultures. The liver is characteristically affected and shows a general parenchymatous degeneration and necrosis; the entire gastrointestinal tract is intensely congested, and numerous hemorrhagic areas are present; the spleen, dark and soft, is rarely much enlarged; the kidneys are swollen and congested; the adrenals are much swollen and intensely red; the lungs are sometimes congested but otherwise normal. In the case of intratesticular inoculation the scrotum and testicle both undergo rapid gangrene. In monkeys no septicemia has been observed, but a violent local reaction—swelling, congestion, sometimes necrosis—follows intradermal inoculation.

Since no microorganism corresponding in character with this one has previously been described, it is regarded as a new species, and because of its presence in material obtained from Peru it has been given

[3] Noguchi, H., *J. Exp. Med.*, 1926, xliii, 515.

the name *Bacterium peruvianum*. The significance of the association of *B. peruvianum* with *Bartonella bacilliformis* deserves further investigation; it is not impossible that the two organisms are introduced into the human body by the same blood-sucking insect.

Cultures of *Bacterium peruvianum, n. sp.*, an organism associated with *Bartonella bacilliformis* in verruga tissues.

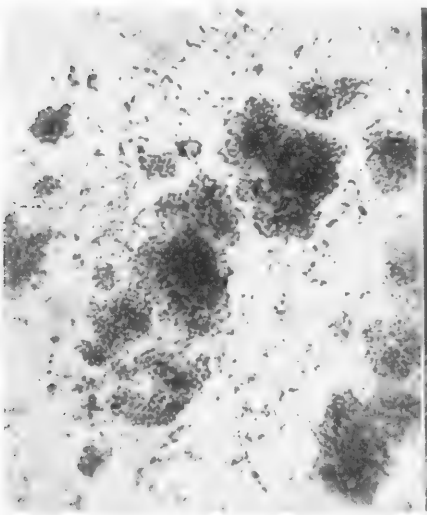

FIG. 1.

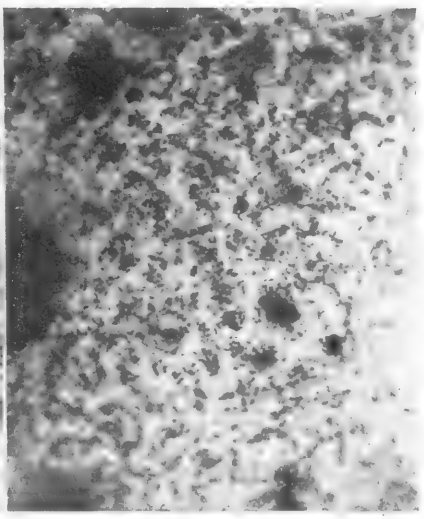

FIG. 2.

Giemsa's stain. × 1000.

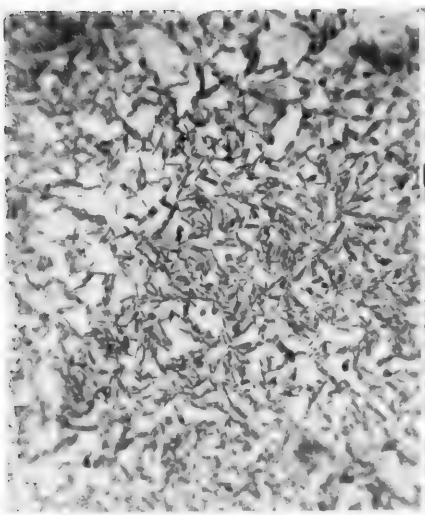

FIG. 3.

Giemsa's stain. × 1000.

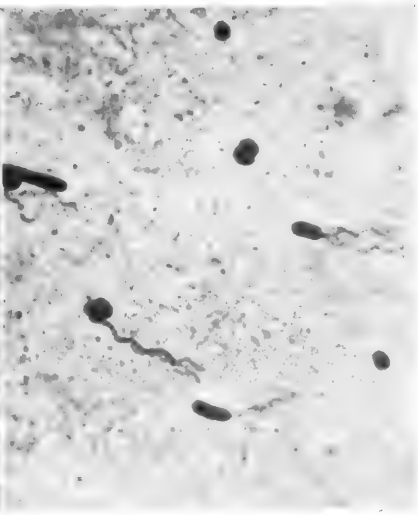

FIG. 4.

Zettnow-Fontana flagella stain. × 2000.

(Noguchi: Etiology of oroya fever. IX.)

UNIV. OF MICH.

ON PRECIPITABLE SUBSTANCES DERIVED FROM BACILLUS TYPHOSUS AND BACILLUS PARATYPHOSUS B.

By J. FURTH, M.D., and K. LANDSTEINER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The present study was primarily undertaken in order to verify certain findings reported on the extraction of precipitable substances of *B. typhosus* with alcohol and ether, but it was extended later to a more general investigation of the precipitable substances of this and allied microorganisms (1).

A number of papers have dealt with the question of lipoids or alcohol-soluble specific substances of typhoid bacilli.

Pick (2) investigating chemically the nature of the specific substances of the *B. typhosus* described one soluble in alcohol which gave no protein and no Molisch reaction and resisted digestion. In concentrated solution it was precipitated by immune sera. Nicolle (3) reported that the agglutinogens of typhoid bacilli are soluble in alcohol and ether but Winterberg (4) was unable to confirm these results. That the lipoids of typhoid bacilli, or even the saponified fats, possess antigenic activity was claimed by Schachenmeier (5) and Stuber (6). Borcic (7), H. Schmidt (8), and Weil and Felix (9), however, failed in their attempts to use alcohol or ether extracts of the typhoid bacilli for immunization. Similar negative results were recorded by Zurugzoglu (10). Very recently Przesmycki (11) has reported that ordinary antibacterial immune sera react specifically with alcoholic extracts of homologous bacilli. Definite statements that bactericidal immune sera develop after the injection of alcohol and ether extracts of typhoid bacilli have been made by Schlemmer (12). So far as we are aware Schlemmer's work has not been repeated as yet.

EXPERIMENTAL.

Bacillus typhosus.—Immune sera were produced by injections of ether extracts of typhoid bacilli according to the directions of Schlemmer. The sera obtained had very weak agglutinating and bactericidal activity when compared with typhoid immune sera obtained in the usual way with whole bacilli.

Attempts to prepare alcohol-soluble precipitable substances from typhoid bacilli by the method of Pick gave no clear-cut results with our material. A saline extract of agar cultures of *B. typhosus* was precipitated with alcohol and both the centrifuged sediment and the supernatant fluid were tested with a precipitating immune serum. It was found that the bulk of the precipitinogen was carried down in the precipitate while the solution contained only a small fraction of the active substance.

Definite results were secured when in place of ether or absolute alcohol 75 per cent alcohol was used for the extraction, in the manner already described for *Vibrio choleræ* (13).

B. typhosus was grown on agar for 24–48 hours at 37°C. The bacilli were centrifuged, washed once with saline and twice with 95 per cent alcohol, and heated for about 2 hours with absolute alcohol (10 cc. per Blake bottle) on the steam bath. The bacilli were collected by filtration on a hot water funnel and extracted twice with boiling 75 per cent alcohol for about 2 hours, the suspension filtered hot, and the filtrate chilled in the ice box. The precipitate formed was dried after washing with 95 per cent alcohol and absolute alcohol and ether. For the precipitin and chemical tests the substance was dissolved in weak alkali and precipitated by acidifying with acetic acid. The procedure was repeated two to three times. This preparation will be referred to as 75 per cent alcohol extract or P1.

When the substance was tested with ordinary typhoid immune sera obtained from rabbits injected with typhoid bacilli heated to 60–62°C. for about 40 minutes, faint reactions only were noticed. We tried therefore to prepare immune sera with the product itself. It was found to possess strong antigenic activity. Two to three injections of 0.2–2 mg. each were sufficient for the production of immune sera which, in contrast to the ordinary typhoid immune sera, precipitated strongly the extracted substance, but agglutinated only feebly suspensions of *B. typhosus* (Table I).

The substance is insoluble in water but soluble in weak alkali. It precipitates out of its solution on acidifying. It gives strong protein reactions. After digestion with trypsin or treatment with antiformin the substance is no longer precipitated by immune sera. On hydrolysis for 5 hours only slight or no reduction of Fehling's reagent was found. The product contains only traces of phosphorus

and traces of lead-blackening sulfur. The N content of the ash-free preparation was 15.7 per cent.

Since the alcohol extract gave definite reactions only with a special immune serum the attempt was made to prepare solutions which would react with the ordinary immune sera. Such active solutions have been obtained previously by various means of extraction, as with saline solution (Pick (2), Weil and Felix (9)), dilute alkali (Zinsser and Parker (14)), antiformin (Altmann and Schultz (15), Krumwiede and Nobel (16)) etc.

We extracted active substance in the following manner:

Washed bacilli previously treated with 95 per cent, hot absolute, and hot 75 per cent alcohol, as described above, were extracted for 2 hours on the steam bath with 2 cc. of .9 per cent saline solution per Blake bottle. After centrifugalization the solution was precipitated with about half the volume 95 per cent alcohol. This crude material was purified to a certain extent by repeated precipitations with acid, and by extraction with 75 per cent alcohol as described below. The product obtained is designated as P2.

Immune sera active for P2 were got by immunization of rabbits with the bacillary residue remaining after the preparation of P1. These sera had a considerable agglutinin titer for live typhoid bacilli.

The product P2 dissolves readily in water and is precipitated by acidulating. It has the reactions and composition of a protein (N 17.2 per cent) and contains only faint traces of phosphorus and traces of lead-blackening sulfur. After hydrolysis for 5 hours only a slight or no reduction of Fehling's reagent was found.

By digestion with trypsin or treatment with antiformin the serological activity, tested by precipitation, was destroyed like that of P1.

That specific substances differing from those just described are present in typhoid bacilli seemed to follow from the important study of Douglas and Fleming (17, *cf.* 11). These authors digested with trypsin typhoid bacilli previously extracted with acetone. Using this material they produced in rabbits immune sera which reacted with the solution resulting from the digestion after removal of an undigested part. The sera had a high bactericidal activity but a weaker agglutinating activity than ordinary immune sera and precipitated more intensely the soluble digestion product. The authors

seem inclined to believe that this precipitable substance is a lower degradation product of proteins.

On repeating[1] the experiments of Douglas and Fleming which deal with the precipitinogen we arrived at confirmatory results. The immune sera obtained had moderate agglutinating power (2000–5000) and only a slight precipitating action for the substances P1 and P2. In conformity with these facts the immune sera for P1 and P2 did not react with the solution resulting from digestion.

When the solution resulting from digestion was made strongly alkaline and precipitated with alcohol a material was obtained with a high content in carbohydrates. Substances with similar properties could be prepared by various methods, *e.g.* as follows:

Typhoid bacilli grown on agar were taken up in N/2 sodium hydroxide solution and kept for about 1½ hours at 37°C. After adding hydrochloric acid till only a slight alkalinity remained and centrifuging, the fluid was precipitated with 1½ volumes of alcohol, the precipitate was redissolved, and after removing some insoluble material and adding hydrochloric acid to make the solution N/10 it was again precipitated with 1½ volumes of alcohol.

This crude product, designated as $C_{typh.}$, gave negative or faint protein reactions, yielded 39.4 per cent reducing sugars after hydrolysis for 5 hours with N/2 hydrochloric acid, and contained N 4.05 per cent (ash-free). Qualitative tests for phosphorous were strongly positive. (A sample obtained after preciptation with barium hydroxide gave, on hydrolysis, about 46 per cent reducing sugars and had a N content of 3.9 per cent.)

Apparently the same substance can be extracted by dissolving the typhoid bacilli with alkaline hypochlorite solution as suggested by Altmann and Schultz, Krumwiede and Nobel.

Typhoid bacilli grown on agar were suspended in saline, and antiformin was added in sufficient amount to cause solution of the bacteria at 55°C. When to this unneutralized solution alcohol was added an active substance separated out. This was suspended in water and after removal of some insoluble material again precipitated with alcohol.

The preparation $C_{typh.,}$ exhibits serological reactions similar to those of the substance of Douglas and Fleming. Its serological activity,

[1] In place of acetone, alcohol was used for the extraction.

unlike that of P1 and P2, is not affected by tryptic digestion and treatment with antiformin (1–10 per cent solution for 30 minutes at 37°C.). The preparation was not precipitated in as high dilutions as apparently similar substances from other bacilli.

The precipitation tests performed with the preparations of *B. typhosus* described are summarized in Table I.

TABLE I.

Precipitation Tests with Various Preparations of B. typhosus.

Preparation	Dilution of antigen	Immune sera prepared with					Normal serum
		Whole bacilli		P1	Bacilli after extraction of P1	Digested bacilli	
		96	31				
P1	1,000	+±	tr.	+++	f.tr.	+	f.tr.
	5,000	tr.	0	++±	tr.	±	0
	25,000	0	0	+	0	0	0
	100,000	0	0	±	0	0	0
	500,000	−	−	0	−	−	−
P2	1,000	+		±	++	tr.	0
	5,000	+		tr.	+++	0	0
	25,000	±		0	++	0	0
	100,000	tr.		0	+	0	0
	500,000	0		−	tr.	−	−
$C_{typh.}$	1,000	++		0	0	+++	0
	5,000	+		0	0	++	0
	25,000	tr.		0	0	±	0
	100,000	f.tr.		0	0	tr.	0
	500,000	0		−	−	f.tr.	−
Agglutinin titer for live bacilli		50,000	20,000	100	10,000	2,000	100 neg.

In this and the following experiments 1 drop of immune serum was added to 0.2 cc. of the diluted antigen and the reactions were read after 2 hours incubation at room temperature.

It seems from these experiments that the three preparations tested are distinctly different as to their serological reactions. This holds particularly for substance C. Between P1 and P2 group reactions occur to a slight degree and both react weakly with an immune serum for C. Since this latter reaction did not disappear after treatment

with antiformin it is very probably due to the presence of C in P1 and P2. With several other preparations the group reactions were more pronounced.

When preparing P1 the bacilli were extracted several times with 75 per cent alcohol. The first and second extracts reacted mainly with sera for P1 whereas later extracts gave also considerable reactions with sera for C. In preparing P2 a similar behaviour was noted. The second saline extract gave a stronger reaction for C than the first. The content in C of the protein preparations could be reduced by repeated precipitations with acid. P2 could be freed to a certain extent from P1 by dissolving in 1 per cent saline solution, adding alcohol to a concentration of 75 per cent, boiling, and filtering hot. P2 was isolated from the insoluble part by dissolving in slightly alkaline water and precipitating with acid.

Bacillus paratyphosus B.—From *Bacillus paratyphosus* B a specific substance was prepared by extraction with 75 per cent alcohol as described above. This preparation contained proteins and a considerable amount of carbohydrates. It was precipitated strongly by the ordinary paratyphoid B immune sera and induced the formation of agglutinins and precipitins when injected into rabbits. It was precipitated to a certain extent by immune sera for the substance P1 of typhoid bacilli, but this reaction disappeared after digestion with trypsin or treatment with antiformin while the reaction with paratyphoid immune sera persisted after such treatment. This behaviour suggested the presence in the alcoholic extract of two specific substances analogous to the preparations P_1 and C from *B. typhosus*. In order to prepare the latter the following method was used.

Bacilli previously extracted with hot absolute alcohol were heated on the steam bath with saline solution for about 1½ hours. The suspension was centrifuged, acidulated, and after the removal of the precipitate by centrifugalization the supernatant liquid was precipitated with alcohol. The precipitation with alcohol was repeated 2–4 times in acid and in alkaline solution.

The substance which will be designated as $C_{paratyph.\ B}$ gave only faint protein reactions. After hydrolysis with N/2 hydrochloric acid for 5 hours 63.8 per cent reducing sugar was found, calculated as glucose. An analysis gave the following figures for ash-free substance: C 43.8 per cent, H 6.5 per cent, N 1.86 per cent, P 2.06 per cent, ash 7.16 per cent. The yield was considerable and amounted to 10–20 mg. per Blake bottle.

Tests for Species Specificity.

The precipitable substances derived from typhoid and paratyphoid bacilli and the corresponding immune sera were tested with a number of other immune sera and antigens respectively (Tables II, *a*, *b*, and III).

TABLE II, *a*.

Precipitation Tests with P1 from B. typhosus and Various Immune Sera.

Dilutions of antigens	Immune sera obtained with									
	B. typhosus			*B. enteritidis*		*B. paratyphosus* B		*V. choleræ*		*Proteus* HX19
	Whole bacilli		75 per cent alcohol extract	Whole bacilli	75 per cent alcohol extract	Whole bacilli	75 per cent alcohol extract	Whole bacilli	75 per cent alcohol extract	Whole bacilli
1,000	+	tr.	−	f.tr.	−	±	−	tr.	0	0
5,000	tr.	0	+++	0	++	f.tr.	+++	f.tr.	0	0
50,000	0	0	+	0	+	0	+	0	0	0
500,000	0	0	0	−	0	−	0	−	−	−

TABLE II, *b*.

Precipitation Test of an Immune Serum for P1 and Crude 75 Per Cent Alcohol Extracts from Various Organisms.

Dilutions of antigens	75 per cent alcohol extract obtained from					
	B. typhosus	*B. enteritidis*	*B. paraty-phosus* B	*B. coli*	*V. choleræ*	*Proteus* HX19
1,000	−	−	−	+	0	0
5,000	+++	++	+	+	0	0
50,000	+	±	tr.	tr.	0	0
500,000	0	0	0	−	−	−

It appears from Table II, *a* and *b*, that the serum against the substance $P1_{typh.}$ gives marked group reactions with the analogous substances of *B. enteritidis*, *B. paratyphosus* B, and *B. coli*, and not with the preparations of the more distant organisms, *V. choleræ* and *Proteus* HX19. This result was confirmed by tests with various immune sera.

There were less pronounced group reactions with the preparation

TABLE III.

Precipitation Tests for Specificity with the Preparations C.

Substances obtained from	Dilutions of antigens	Immune sera prepared with									
		B. typhosus digested bacilli		*B. enteritidis* G whole bacilli		*B. paratyphosus* B whole bacilli		*Proteus* HX19 whole bacilli		*V. choleræ* whole bacilli	
B. typhosus	1,000	++±	++++	+	++±	0	0	0	0	0	0
	5,000	+±	+++	±	+±	0	0	0	0	0	0
	25,000	±	++	f.tr.	tr.	0	0	0	0	0	0
	100,000	tr.	+±	0	0	0	0	0	0	0	0
	500,000	0	tr.								
		(5,000)		(10,000)		(1,000)					
B. enteritidis G	1,000	++	++++	±	++±	0	0	0	0	0	0
	5,000	+±	+++	+	++±	0	0	0	0	0	0
	25,000	±	++±	+±	+++	0	0	0	0	0	0
	100,000	0	f.tr.	+	+++	0	0	0	0	0	0
	500,000	0	0	±	++						
				(5,000)		(100)					
B. paratyphosus B	1,000	+±	++	0	tr.	+±	++±	0	0	0	0
	5,000	tr.	+	tr.	±	+±	++±	0	0	0	0
	25,000	0	tr.	±	+	++	+++	0	0	0	0
	100,000	0	0	tr.	+	+±	++++	0	0	0	0
	500,000			f.tr.	±	tr.	++±				
		(1,000)*		(1,000)		(50,000)					

Proteus OX19	1,000	0	tr.	0	0	0	0	++	+++	0	0
	5,000	0	0	0	0	0	0	++++	+++	0	0
	25,000	0	0	0	0	0	0	++++	++++	0	0
	100,000	0	0	0	0	0	0	++±	++++	0	0
	500,000							+	++		
								(2,000)			
V. choleræ	1,000	tr.	tr.	0	0	0	0	0	0	++++	++++
	5,000	0	0	0	0	0	0	0	0	++++	++++
	25,000	0	0	0	0	0	0	0	0	+++	+++
	100,000	0	0	0	0	0	0	0	0	+±	++±
	500,000									tr.	+
										(20,000)	

First readi .g after 2 hours room temperature, the second after standing overnight in the ice box. The figures indicate the aggluti .in titre.

* Very weak reactions also in high concentrations of serum.

P2. $P2_{typh.}$ was not acted upon in the dilutions tested by two common immune sera against *B. enteritidis* and gave a weak reaction with only one of two common immune sera against *B. paratyphosus* B. A moderate reaction became evident when a serum very active against $P2_{typh.}$ was tested with a crude P2 preparation of *B. paratyphosus* B, and a faint reaction occurred with a similar preparation of *Proteus* HX19.

The reactions involving the species specificity of the substances C are summarized in Table III. The substance C of *V. choleræ* has already been described (13). C_{OX19} was obtained in a similar manner from *Proteus* OX19.[2] This latter product gave negative or faint protein reactions and had a carbohydrate content of 57.3 per cent after hydrolysis for 5 hours. A substance apparently with the same serological and chemical properties could be prepared from *B. proteus* HX19 with about the same yield as that got from *Proteus* OX19. C of *B. enteritidis* was prepared in the same manner as $C_{typh.}$ The yield of reducing sugars after hydrolysis of this substance was 56.89 per cent (calculated as glucose for ash-free substance).[3]

In tests with the substances C showing strong reactions, the precipitates appear generally in heavy flakes or membranes unlike the more fluffy precipitates of proteins as has already been observed by Avery and his coworkers.

DISCUSSION.

The investigations reported may be regarded as an initial step in the study of the antigens of typhoid bacilli and related organisms. The work should be extended in various directions with special reference to the purification of the isolated products, and the rôle they play in the group reactions of the related species. Methods of preparation should be studied, a search made for additional active substances, and various strains of the same organism examined.

The active substances thus far prepared fall into two groups differentiated sharply by their behaviour towards trypsin and alkaline

[2] A report on specific polysaccharides in *Proteus* HX 19 has been made recently by Przesmycki (18).

[3] An antigenic solution of *B. enteritidis*, containing traces of proteins and much carbohydrate was dealt with recently by Branham and Humphreys (19).

hypochlorite solution (antiformin). Those of one group (P) are easily destroyed by these agents and behave in general like proteins, while the others (C) are resistant to trypsin and considerably so to antiformin and yield a large quantity of reducing sugar on hydrolysis. There was no definite evidence in our studies with *B. typhosus* of the existence of lipoids as assumed by Schlemmer and others.

The presence in bacilli in general and in typhoid bacilli especially of specific non-protein substances which do not induce antibody formation has been emphasized by Zinsser and Parker (14). An indication of the existence of such products can be found in the paper of Pick although he considered the possibility that his substances were disintegration products of proteins. A similar view was held by Douglas and Fleming (17) concerning their substances obtained by digestion.

In the light of the well known studies of Avery and Heidelberger it seemed likely that the specific component in the products of the above authors may belong to the group of specific carbohydrates, a view substantiated by our findings. The immune sera obtained by us following the directions of Douglas and Fleming reacted on our substance C which is almost free from proteins and rich in carbohydrates. This preparation C will probably prove to be a hapten in our terminology, *i.e.*, a specific substance devoid of antigenic activity.

Substances belonging apparently to the same group, that is to say yielding much sugar on hydrolysis and practically free from protein, were also found in *B. paratyphosus* B, *B. enteritidis*, and *Proteus* HX19 and OX19.[2]

On cross-testing the various substances of the group C with the corresponding immune sera the strongest group reaction occurred between *B. typhosus* and *B. enteritidis*, the homologous reactions being more intense. The substance of *B. paratyphosus* B was precipitated by the sera for *B. typhosus* and *B. enteritidis* though not very intensely. It is remarkable that the reciprocal reactions, namely those of the paratyphoid B serum with the substances from *B. typhosus* and *B. enteritidis* were entirely negative. For an understanding of these relations further investigations are required, involving also absorption tests. With the substances and sera of microorganisms not belonging to the typhoid groups negative or only faint reactions took place.

Observations similar to ours were made by Krumwiede and Nobel who found strong reactions of typhoid immune sera with antiformin extracts of *B. pullorum* and *B. sanguinarium* and faint reactions with extracts of *B. paratyphosus* B. Probably the active substances in the antiformin extracts correspond to our preparations C.

The tests with the preparations P1 and P2 show that the proteins of a given bacillus may be sharply differentiated by serological reactions in the same way as the proteins of an animal species (20) or those of yeasts (21). No effort has yet been made to fractionate our active protein preparations or to isolate other precipitable proteins.

The high antigenic power of the bacterial proteins is remarkable (22) and it may serve to explain some of the statements published on the antigenic activity of preparations from bacilli that are apparently protein-free. In view of the antigenic capacity of P1 it seems significant that the immune sera formed after injection of whole bacilli heated to 60 or 100°C. have ordinarily only a weak action on P1, so that it is necessary to immunize with the substance itself if one desires to obtain potent precipitins for P1. This phenomenon could be explained either by a change of the precipitable substance through the action of alcohol, or more readily by the masking of its antigenic activity in the original complex. To such an effect one may ascribe also the fact that the sera active for the various precipitable fractions, especially the P1 serum, are considerably less agglutinative than the common immune sera. Examples of both phenomena, namely of the failure of an immune serum for a complex antigen to react with the components of that antigen and of the failure of a serum for one component to react with the complex were encountered in the study of blood antigens (23). Avery and his coworkers (24) have described similar findings in their work on pneumococci.

By absorbing a common typhoid immune serum with bacilli treated with alcohol a fluid was procured which is supposed to contain the flagellar agglutinins (Smith and Reagh (25)) and indeed agglutinated live typhoid bacilli to a high titer and also suspensions containing flagellæ (Orcutt (26)). The fluid failed to precipitate any of our precipitable substances. Similarly an immune serum for *Proteus*

HX19 after absorption with *Proteus* OX19 did not precipitate the substance C_{x19} although it agglutinated intensely *B. proteus* HX19 (not *Proteus* OX19). Thus there is no proof as yet of a connection of these precipitable substances with the so called "labile" agglutinogen.

SUMMARY.

Attempts to confirm certain statements that ether-soluble specific substances can be obtained from *B. typhosus* have lead to negative results.

Two serologically active protein substances and another that was non-protein have been separated from *B. typhosus*. The first two are not resistant to tryptic digestion or to treatment with alkaline hypochlorite solution whereas the third resists both. One of the proteins couid be extracted with 75 per cent alcohol.

Specific precipitable substances reacting like the non-protein substance of *B. typhosus* and containing large amounts of carbohydrates have been prepared from *B. paratyphosus* B, *B. enteritidis*, and *Proteus* HX19 and OX19. Observations on the serological behaviour of these preparations are described.

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